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Plasma Levels of β-Thromboglobulin and Platelet Factor 4 in Relation to the Venous Platelet Concentration

Jack Kutti, Soodabeh Safai Kutti, Charles G. Zaroulis and Robert A. Good

Hematology Service, Memorial Sloan-Kettering Cancer Center, New York, N.Y.

Key Words. β-Thromboglobulin · Platelet factor 4 · Platelet transfusion · Platelets

Abstract. The plasma levels of β-thromboglobulin (β-TG) and platelet factor 4 (PF-4) were determined in patients with various hematologic malignancies, and the results were related to simultaneously determined venous platelet counts. All studied patients were in a steady state. The plasma β-TG concentrations were determined on 69 occasions and the values ranged from 0 to 82 ng/ml. In 33 instances, the venous platelet count was $<25 \times 10^9$/l and in two thirds of these samples β-TG was undedectable. The highest values for plasma β-TG were found in patients with the highest venous platelet counts. A highly significant correlation ($r = 0.77$; $p < 0.001$) between the values for plasma β-TG and venous platelet count was present. The plasma concentrations for PF-4 ranged from 0 to 50 ng/ml. Similarly there was a highly significant relationship ($r = 0.78$, $p < 0.001$) between the values for PF-4 and venous platelet concentration. We conclude, if the plasma levels of β-TG and PF-4 are used as markers of platelet activation *in vivo*, it is necessary to simultaneously consider the platelet concentration in the collected blood.

Introduction

β-Thromboglobulin (β-TG) and platelet factor 4 (PF-4), two platelet-specific proteins contained within the α-granule [9, 10] are liberated into the plasma during the platelet release reaction. Lately considerable interest has focused on the measurement of the plasma concentrations of β-TG and PF-4, because elevated plasma values of these proteins are considered to be specific indicators of platelet activation in a variety of clinical situations [2, 4, 5, 7, 8, 12, 14].

Currently clinical investigators relate the results of the plasma β-TG and PF-4 determinations to those values obtained from healthy subjects. Recently we observed that the plasma β-TG levels in thrombocytopenic patients with acute leukemias frequently are considerably lower than in normal subjects [11]. Further, in a group of leukemic patients with variable venous platelet counts a significant positive correlation between the values for plasma β-TG and venous platelet concentration was observed. This relationship was present despite the fact that

patients with fever septicemia, and disseminated intravascular coagulation were included in the analysis. Since β-TG and PF-4 are markedly homologous in structure [1] but vary as regards physical and biological properties, it could well be anticipated that a similar relationship between the plasma values for PF-4 and venous platelet counts exists. In the present study we attempted to establish steady state values for plasma β-TG and PF-4 in patients with different hematologic neoplasms.

Materials and Methods

Patients

A total of 43 patients (24 males and 19 females, aged 17–77 mean 41 years) with acute leukemias and various myeloproliferative and lymphoproliferative disorders were investigated. The majority of the patients (83%) had acute myeloblastic or lymphoblastic leukemias, 2 patients had acute *myelomonoblastic leukemia and 1 patient each* had hairy cell leukemia, blastic chronic granulocytic leukemia, mycosis fungoides, diffuse histiocytic lymphoma, and nodular histiocytic lymphoma. All *studied patients were receiving antileukemic or* antilymphomatous chemotherapy at the time of investigation except for the patient with hairy cell leukemia. Patients with acute promyelocytic leukemia were excluded from this study At the time of blood collection all patients were afebrile, there were no clinical signs of active thromboembolic disease, and no laboratory evidence of disseminated intravascular coagulation. The leukemic patients had received many platelet transfusions prior to and during the period of this study although the patients with lymphoproliferative disorders had received few if any platelet transfusions before or during this investigation. The plasma β-TG values were determined on 69 occasions, and in 36 instances the plasma PF-4 concentrations were concomitantly measured. In association with each determination of β-TG and PF-4 venous blood was also collected for platelet counting using phase microscopy [3]. Informed written consent was obtained from all subjects participating in the study according to the principles of the Declaration of Helsinki.

Blood Sampling

Blood was obtained from an antecubital vein using a 19-gauge butterfly needle· great care was taken to perform a nontraumatic venipuncture using minimal or no venous occlusion. The first 2–3 ml of blood were always discarded. For the determination of β-TG about 3 ml of blood was collected into a 5-ml plastic syringe and immediately 2.5 ml were transferred into a precooled plastic tube containing EDTA and theophylline. Promptly thereafter blood was collected for determination of PF-4 Blood was obtained using a standard 10-cm^3 vacutainer tube containing liquid EDTA. Immediately after collection of blood, each tube was gently inverted three times and was quickly placed in an ice water bath.

Preparation of Plasma

The anticoagulated blood was centrifuged at 1,800 *g* for 30 min at +4 °C within 30 min of collection. After centrifugation, 0.5 ml of plasma was obtained 5–10 mm below the upper plasma layer using a 26-gauge needle attached to a disposable 1-ml tuberculin syringe [11]. Specific caution was taken not to tilt or tap the tubes or otherwise disturb the top plasma layer [13] The plasma samples were frozen at −20 °C and assayed within 2 weeks of separation.

Assay of β TG and PF-4

The plasma concentrations of β-TG and PF-4 were measured by radioimmunoassays. The β TG radioimmunoassay was purchased from Amersham (Arlington Heights, Ill.)· the PF-4 radioimmunoassay was a kind gift from Dr *E. J Fedor* Diagnostic Division, Abbott Laboratories (North Chicago, Ill.).

Results

Plasma Levels of β-TG (fig. 1a)

For the 69 samples analyzed the plasma concentrations of β-TG ranged from 0 to 82 ng/ml. The venous platelet count was $< 25 \times 10^9$/l in 33 instances and in 22 of these samples immeasurable values for

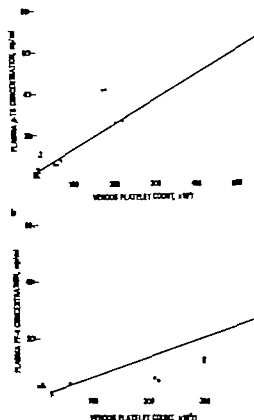


Fig. 1. Relationship between plasma β-TG concentration and venous platelet count (n = 69) in group of patients with various malignant hematologic disorders and between plasma PF-4 concentration and venous platelet count (n = 36) in group of patients with various malignant hematologic disorders (b).

β-TG were present. In these 33 samples the highest value for plasma β-TG was 12 ng/ml. Patients with the highest venous platelets counts had the highest values for plasma β-TG concentrations. A highly significant relationship ($r = 0.77$, $p < 0.001$) between the values for plasma β-TG and venous platelet count was present, and the equation for the regression line was: $y = 0.12x + 1.37$

Plasma Levels of PF-4 (fig. 1b)

The plasma concentrations for the 36 PF-4 determinations ranged from 0 to 50 ng/ml. In patients with venous platelet counts $< 25 \times 10^9/l$ undetectable levels for PF-4 were present in 6 of 16 cases, and in those instances the values for PF-4 were always ≤ 4 ng/ml. The highest plasma values for PF-4 were detected in patients with the highest venous platelet concentrations. A highly significant correlation ($r = 0.78$, $p < 0.001$) between the values for plasma PF-4 and venous platelet count was shown to be present. The equation for the regression line was. $y = 0.97x - 0.37$

In the 36 instances when the plasma levels of β-TG and PF-4 were measured concomitantly as an average the plasma concentration for β-TG was 1.6 times greater than the concentration for PF-4

Discussion

The present study has demonstrated that there is a highly significant positive relationship between the plasma concentrations of β-TG as well as PF-4 and the platelet count in the blood collected for analysis. Indeed, this concordance is anticipated since the centrifugation procedures currently used for preparation of plasma for β-TG and PF-4 analysis do not provide plasma free of platelets [13]. *Wolf* [15] pointed out that ultracentrifugation is required to obtain plasma free of platelets and platelet dust. As can be seen in figure 1a and b, both regression lines almost intersect the origin. Clearly this observation implies that under steady

state conditions undetectable or very low plasma concentrations of both proteins are expected to be found in severely thrombocytopenic patients. Consequently in such patients plasma β-TG and/or PF-4 concentrations within the range of normal controls must be considered as highly abnormal. Recently *Dawes et al* [6] demonstrated that the half-life of β-TG in the circulation was about 100 min, however the plasma clearance of PF-4 was so rapid that its half-life could not be estimated. Therefore, it seems likely that the major portion of the plasma β-TG as well as the PF-4 concentration, encountered in the steady state patient (or the healthy subject) with normal platelet counts, is contributed by a small number of residual platelets in the separated plasma rather than from true freely circulating platelet-specific protein.

At the time of blood collection all patients in the present study were considered to be in a steady state. Nevertheless, a wide distribution of individual plasma values for β-TG and PF-4 over given venous platelet counts was obtained (fig. 1a, b) This finding could be due to differences in the patients clinical status at the time of blood collection i.e., in some cases a subclinical activation of the coagulation system might have been present. However despite major efforts to strictly standardize the sampling and processing techniques (each platelet specific protein radioimmunoassay had a coefficient of variation of less than 10 7%), it could well be that minor differences in the above procedures resulted in the differing values between individuals for β TG and PF-4 It should also be stated that if a group of 42 normal controls were included in the analyses the slopes of the regression lines were unchanged. Although, in the present study only patients with acute leukemias, lymphoproliferative, and myeloproliferative disorders were investigated, it seems probable that the present data are applicable for other steady state clinical situations. Consequently it is evident that whenever the values of β TG and PF-4 are used as indicators of platelet activation and release reaction *in vivo* it is mandatory to consider the concentration of platelets in the collected blood.

Acknowledgements

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Charles G Zaroulis, MD
Memorial Sloan-Kettering Cancer Center
1275 York Avenue,
New York, NY 10021 (USA)

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Charles G. Zaroulis, MD,
Memorial Sloan-Kettering Cancer Center
1275 York Avenue,
New York, NY 10021 (USA)

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Increased Platelet Aggregation Induced by Glucagon Administration

Stephen J Proctor Terry F Davies Alan Oxley Peter Jones and Alan Heath

Endocrine Unit, Department of Medicine and Department of Haematology Royal Victoria Infirmary Newcastle upon Tyne

Key Words. Primary aggregation Platelet Glucagon Aspirin

Abstract. The effect of 1 mg of intravenous glucagon on platelet aggregation has been investigated in 12 normal subjects pre treated with salicylate. All subjects demonstrated the expected inhibition of collagen-induced secondary aggregation but retained the normal adenosine diphosphate (ADP)-induced primary aggregation phase 18 h after the salicylate therapy (600–1,200 mg) Subsequent administration of glucagon caused a significant increase in the ADP induced primary aggregation phase in the 12 subjects. This data indicates that glucagon increases the reactivity of platelets to ADP and may help to explain the common clinical association of raised plasma glucagon increased platelet aggregation and vascular disease.

Introduction

Human platelets have a number of pharmacological receptors and are responsive to a variety of hormones. Many such agents influence the adenylate cyclase-cyclic adenosine 3',5' monophosphate (cAMP) system which is present within human platelets [*Salzman and Neri* 1969] but the precise role of cAMP in platelet function is not fully elucidated.

Glucagon is a potent stimulator of plasma cAMP increasing basal levels thirty fold [*Davies et al.*, 1976] and may therefore alter platelet function. In order to determine the influence of glucagon on the primary phase of aggregation we investigated its effect in normal subjects before and after pre treatment with aspirin.

Methods

12 normal subjects were studied aged 21–55 years including 8 males and 4 females. All had refrained from any drug treatment for the previous 4 weeks. All were pre-treated with aspirin, 600–1,200 mg, 18 h prior to testing. 9 of the subjects were also tested with glucagon prior to the aspirin therapy 2 of the subjects had further tests on separate occasions and received the diluent only

All subjects were fasted overnight and tested between 9.0 and 10.0 a.m. After withdrawal of basal venous samples, 1 mg of glucagon (Eli Lilly)

in 1 ml of diluent (1.6% glycerol and 0.2% phenol) diluted to 10 ml with sterile water was given intravenously over 1 min. Venous blood was withdrawn at 15, 30, 45 and 60 min thereafter. These samples were obtained by multiple venepuncture. Samples taken via indwelling catheters showed reduction in platelet number and aggregation studies were unreliable.

The following investigations were performed on each blood sample obtained: The full blood count and platelet count were determined by Coulter Counter Model S and Coulter Thrombo Counter. Platelet aggregation was assessed using adenosine diphosphate (ADP- final concentration 0.5 μM) and collagen (4 μg added) and EEL 169 Aggregometer and flat bed recorder by the standard Born technique. All aggregation studies were performed at 37 °C. Blood for platelet studies was collected into 3.8% citrate solution and centrifuged at 50 g to yield platelet plasma. The one-stage prothrombin time, partial thromboplastin time with kaolin, fibrinogen [*Miller et al.*, 1971] and fibrinogen degradation product estimation (ThromboWellotest) were carried out on platelet-poor plasma. Blood glucose was estimated on a Technicon Autoanalyser. Plasma cAMP was measured by using simplified competitive protein binding assay which required no previous plasma extraction [*Latner and Prudhoe* 1973]. Blood for the plasma cAMP assay was collected into cooled heparinised tubes containing 32 M theophylline solution and stored at −70 °C until assayed.

Results

The 2 control subjects who received diluent and water alone showed no effect upon any of the parameters assessed. In the tests using glucagon the platelet count and

Table I

Case No	Pre-glucagon OD max (P) mm	Post-glucagon							
		15 min		30 min		45 min		60 min	
		OD max (D)	D–P (X)	OD max (D)	D–P (X)	OD max (D)	D–P (X)	OD max (D)	D–P (X)
1	60	80	20	132	72	160	100	130	70
2	94	130	36	122	28	93	− 1	125	31
3	49	100	51	86	37	74	25	66	17
4	92	106	14	148	56	119	27	94	2
5	40	86	46	77	37	71	31	63	23
6	4	44	20	36	12	35	11	45	21
7	37	62	25	40	3	35	− 2	34	− 3
8	103	110	7	91	−12	92	− 11	98	− 5
9	94	92	− 2	92	− 2	86	− 8	88	− 6
10	65	69	4	63	− 2	75	10	69	4
11	68	78	10	66	− 2	73	5	73	5
12	105	110	5	98	− 7	108	3	120	15

P = Optical density maximum (OD max) pre-glucagon of primary aggregation phase following pre-treatment with aspirin. D = optical density maximum at various post-glucagon; X = difference in optical density measured by the degree of aggregation post-glucagon infusion.

Patients 1–7 showed marked response, patients 8–12 showed small response at the dose of ADP used.

coagulation indices remained unchanged throughout. All subjects demonstrated the expectant rise in blood glucose (1 83 ± 0.39 mmol/l at 30 min) and plasma cyclic cAMP (498 ± 40 nm/l at 15 min) af ter intravenous glucagon

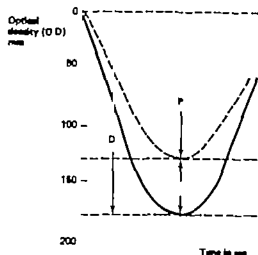


Fig. 1. Diagrammatic representation of aggre gation curve indicating methods of measuring maximum change in optical density — = Pre glucagon curve; — = post-glucagon. P = OD max pre glucagon· D = OD max at various times X = D-P – change in OD at various times post glucagon.

In the nine glucagon tests performed prior to aspirin administration, 8 subjects showed irreversible aggregation with the dose of ADP used (0.5 *μM*) and no effect by glucagon on the aggregation was demonstrated. This indicated that the effect of glucagon was *masked when the platelet release* reaction was not blocked

1 of the male subjects repeatedly demon strated an increase in the primary phase of ADP induced aggregation after the glucagon before salicylate therapy The reason for this was that this subject showed no sec ondary aggregation at the concentration of ADP used (patient 3).

12 subjects treated with aspirin had complete inhibition of collagen induced aggregation and absence of the secondary phase of ADP induced aggregation. With 0.5 *μM* ADP at the time of testing, 7 of these 12 subjects had a marked increase in the primary phase of aggregation measured by maximal change in optical density at var ious times during the 1st hour following glucagon (table I) 5 subjects showed a less marked effect at the concentration of ADP used. As sensitivity to ADP does vary be-

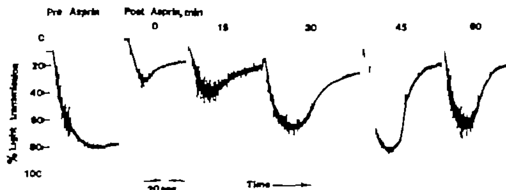


Fig. 2. The pre-aspirin curve using 0.5 *μM* ADP shows complete irreversible aggregation. The same ADP concentration was used throughout the experiment. Following aspirin administration at time 0 the primary aggregation phase is abolished. Intravenous glucagon is given over 20 sec. There is a progressive increase in the degree of primary aggregation reaching a maximum at 45 min. This effect of glucagon can be seen only when aspirin is used to inhibit secondary aggregation.

tween individuals, different concentrations may be required to show maximal effect in some subjects. In the analysis the respon ders and apparent 'non-responders will be considered together

Analysis of Results

The change in degree of aggregation was assessed by a method modified from *Holdrinet et al.* [1969] as seen in figure 1 The preglucagon maximum optical density (OD max) was designated P The OD max post glucagon was designated D D–P = difference caused by glucagon infusion (X). The time (T) to the maximum OD change was not significantly different at the various times post-glucagon.

Statistical Analysis

A comparison of the pre-glucagon OD max and post-glucagon OD max at 15 min post-glucagon using a Wilcoxon signed rank test in the 12 subjects showed a significant increase in amplitude in the level OD max ($p < 0.1$) This effect corresponds to the time of maximal plasma cyclic AMP 7 out of the 12 subjects had a change in OD max of at least 20 U the most marked changes being for subject 1 (fig. 2). In this subject the maximal response was seen at 45 min post-glucagon, indicating the variable re sponse in different subjects. At 60 min, 7 subjects showed a return to the pre-glucagon configuration. Samples were not taken in the remaining 5 subjects beyond 60 min to assess the total duration of the effect.

Discussion

We have shown that glucagon increases the sensitivity of platelets to ADP-induced primary aggregation in normal individuals. This effect was demonstrated to a variable extent in 12 subjects who had been pre treated with aspirin and who subsequently failed to demonstrate the secondary aggre gation phase. We consider it highly unlikely that there was any pharmacological interac tion between glucagon and aspirin to ac count for the effects seen as the experiments were performed when aspirin was cleared from the circulation at 12–18 h (half-life of aspirin in circulation is 3–6 h).

Salicylate achieves its effect on platelets by prevention of the granule release reac tion during the secondary phase of aggregation by its effect on prostaglandin synthe tase and hence, inhibition of platelet prostaglandin production [*Flower* 1975]. This effect was best demonstrated by the lack of collagen-induced aggregation in the aspirin-treated subjects. This irreversible secondary aggregation phenomenon did not return following glucagon infusion suggesting that the hormone does not act on the platelet by re versing the effect of aspirin upon the release reaction.

A number of hormones are known to in fluence platelet behaviour *in vitro*. Catecholamines induce aggregation [*O'Brien* 1963] whereas the prostaglandins are potent inhibitors [*Kloeze* 1967] Arginine and lysine vasopressin are powerful inducers of human platelet aggregation [*Haslam and Rosson*, 1971] and somatostatin has been shown to reduce ADP-induced platelet aggregation in normal human subjects [*Besser et al.*, 1975]

Because of the report that phenol, which is present in the glucagon diluent, may itself influence platelet aggregation [*Zweifler and Sanber* 1969] ⌞ of our subjects also received the diluent only on a separate occasion but no detectable abnormality oc curred.

coagulation indices remained unchanged throughout. All subjects demonstrated the expectant rise in blood glucose (1 83 ± 0.39 mmol/l at 30 min) and plasma cyclic cAMP (498 ± 40 nm/l at 15 min) after intravenous glucagon.

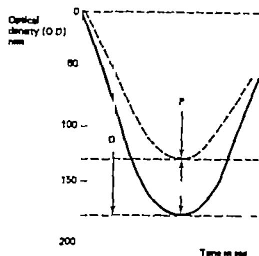


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1 of the male subjects repeatedly demonstrated an increase in the primary phase of ADP induced aggregation after the glucagon before salicylate therapy The reason for this was that this subject showed no secondary aggregation at the concentration of ADP used (patient 3)

12 subjects treated with aspirin had complete inhibition of collagen induced aggregation and absence of the secondary phase of ADP induced aggregation. With 0.5 μM ADP at the time of testing, 7 of these 12 subjects had a marked increase in the primary phase of aggregation measured by maximal change in optical density at various times during the 1st hour following glucagon (table I) 5 subjects showed a less marked effect at the concentration of ADP used. As sensitivity to ADP does vary be-

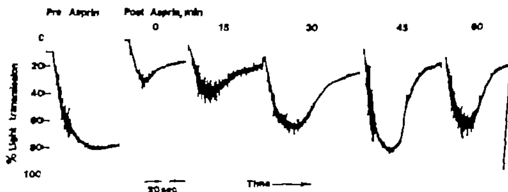


Fig. 2. The pre-aspirin curve using 0.5 μM ADP shows complete irreversible aggregation. The same ADP concentration was used throughout the experiment. Following aspirin administration at time 0 the primary aggregation phase is abolished. Intravenous glucagon is given over 20 sec. There is a progressive increase in the degree of primary aggregation reaching a maximum at 45 min. This effect of glucagon can be seen only when aspirin is used to inhibit secondary aggregation.

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Dr S. J Proctor Department of Haematology
Royal Victoria Infirmary
Newcastle upon Tyne (England)

Glucagon has previously been shown to have no effect upon platelet aggregation behaviour when given as a continuous infusion of 70 μg/kg over $1^1/_2$ h to 5 normal volunteers [*Mannuci and Pareti* 1974]. Our results suggest that in the higher dose we have used, glucagon has a direct effect upon platelets. The way in which glucagon exerts this effect upon platelets is not clear. The maximum increases in both plasma cAMP and glucose occur at 15–30 min, corresponding to the effect of glucagon upon aggregation. Oral glucose in the dosage used in a standard glucose tolerance test (50 g) will reverse the inhibited secondary phase of aggregation after aspirin [*Sagel et al.* 1975]. We have shown no effect upon this secondary phase, and it is therefore unlikely that glucose itself is responsible.

The plasma cAMP response is similar to previous studies in normal subjects in our laboratory [*Davies et al.* 1976]. Intravenous cAMP and dibutyryl cAMP reduce ADP induced platelet aggregation both *in vitro* [*Salzman and Levine* 1971] and *in vivo* [*Zweifler and Sanbar* 1969] affecting both primary and secondary aggregation but only after doses which would produce greater plasma levels than our subjects achieved. Glucagon itself has been shown to stimulate cAMP *in vitro* [*Zieve and Greenough* 1969] but the significance of this remains uncertain. It is possible that high dose glucagon can, in some way, prime the platelet membrane receptors involved in the ADP reaction or influence the membrane in some structural way so revealing more ADP receptors.

Excess glucagon release [*Gerich et al.*, 1975] and raised plasma glucagon levels in diabetes mellitus are also associated with increased platelet aggregation [*Sagel et al.*, 1975] and this may apply in other disease states such as renal failure [*Bilbrey et al.* 1974] and after severe burns [*Wilmore et al.* 1974]. Whether this raised glucagon is responsible for the increased incidence of vascular disease and venous thrombosis in these situations remains an interesting speculation but the association of increased platelet sensitivity to ADP in diabetic peripheral neuropathy is further evidence for this suggestion [*O'Malley et al.* 1975]. High dose glucagon has been associated with pulmonary emboli in dogs [*Sheddon*, 1971] and prolonged intravenous infusion in man has caused peripheral venous thrombosis [*Weitzel et al.* 1973]. Glucagon decreases gastric mucosal haemorrhage induced by aspirin [*Lin and Warrick*, 1974] and stress induced acute haemorrhagic gastritis is improved by intravenous glucagon [*Guth et al.* 1975]. An effect upon platelet aggregation would help to explain these findings and may have therapeutic implications.

We would stress that aspirin in these experiments was used simply as an experimental block to the release reaction and we consider the glucagon effect completely independent of the pharmacological effects of aspirin on the platelet. Of major practical importance is the potential role of aspirin as a tool in experimental systems where investigators wish to assess the effect of various agents *in vivo* or *in vitro* on primary aggregation which otherwise would be masked by the release reaction.

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Dr. S. J Proctor Department of Haematology
Royal Victoria Infirmary
Newcastle upon Tyne (England)

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Effects of Low-Dose 'Factor VIII Inhibitor Bypassing Activity (FEIBA)' in Resistant Haemophilia

Hugo Zwahlen and Eugene A. Beck

Central Haematology Laboratory, Inselspital, and School of Medicine, University of Berne, Berne

Key Words. Factor VIII · Factor VIII inhibitor bypassing activity · Haemophilia · Inhibitors against factor VIII

Abstract. Possible effects and side-effects of 'factor VIII inhibitor bypassing activity (FEIBA)' were tested on 7 haemophiliacs with high titre antibody to factor VIII (resistant haemophilia). FEIBA was administered both to hospitalised patients and as part of a home therapy programme. Serious side-effects, attributable to FEIBA, were one episode of hypersensitivity and, possibly, hepatitis. Our preliminary data suggest that early injection of FEIBA, using considerably lower doses than suggested by the manufacturer, may shorten the duration of immobilisation of haemophiliacs with inhibitor against factor VIII in comparison with infusion of factor VIII and/or supportive care alone, at least following musculoskeletal bleeding. A decrease or complete disappearance of the inhibitor was observed in all patients receiving FEIBA alone.

Introduction

Formation of inhibitors (antibodies) capable of neutralising the therapeutic effect of transfused factor VIII, is a major limitation in the management of haemophiliacs. Therapeutic regimens for treatment of patients with high-titre antibody directed against factor VIII include immunosuppression [8], substitution therapy with high doses of factor VIII [13], or the still debated infusion of activated prothrombin complex [5]. Recently, an as yet poorly defined activated plasma product has been developed which carries the descriptive name 'Factor Eight Inhibitor Bypassing Activity' (FEIBA®) [for review see 6]. We present our preliminary experience gained on possible effects of FEIBA in haemophiliacs with high-titre antibodies against factor VIII. Our results suggest that FEIBA, even at relatively low doses, may reduce the morbidity of such patients, at least in comparison with supportive care alone.

Materials and Methods

Lyophilised cryoprecipitate (approximately 500 units factor VIII per bottle) or intermediate purity factor VIII concentrate was obtained from the

Swiss Red Cross. Fraction R, later designated as FEIBA, was given to us for clinical testing by Immuno, Vienna.

Coagulation Tests

Determinations of the partial thromboplastin time and a one-stage factor VIII assay were performed as previously described [3]. Inhibitors against factor VIII were measured in the same test system, whereby normal plasma was incubated with an equal volume of patient plasma for 15 min, 1 inhibitor unit corresponds to decrease of 0.15 units factor VIII per milliliter in the incubation mixture.

Patients

Preliminary tests were carried out on 7 patients with severe haemophilia who developed an inhibitor against factor VIII (so-called resistant haemophilia) [6]. Informed consent was obtained from adult patients or the parents of children before administering fraction R or FEIBA.

Preliminary Study on Hospitalised Patients

Initially fraction R or FEIBA was administered during hospitalisation under strict medical surveillance. This attitude was particularly prompted by one instance of severe serum sickness in 1 adult patient who also did not support transfusion of any other blood product. Furthermore, positive ethanol gelation test was repeatedly observed up to several hours after injection of FEIBA, suggesting that fibrin formation was taking place in the circulating blood. However using the lower limit of FEIBA dosage, indicated by the producer we did not encounter significant hypofibrinogenemia and or thrombocytopenia.

Of the 7 patients, who initially entered clinical evaluation, 3 could not be studied over prolonged period of time: 1 patient died following perforating psoas bleeding which could not be stopped by infusion of vast amounts of human and porcine factor VIII nor by FEIBA infusions; 2nd patient was already mentioned to exhibit hypersensitivity following the first injection of FEIBA, and the 3rd patient did not properly respond to FEIBA when he developed postoperative haemorrhage following tooth extraction. Bleeding subsided, however promptly following infusion of factor VIII. This patient turned out later to be low responder in terms of formation of anti-factor VIII antibody

Protocol for Home Treatment

The remaining 4 patients (2 adults and 2 children) were put on the following regimen which, in principle, corresponds to home treatment with factor VIII of our haemophiliacs: the patient decided primarily whether or not he had bleeding. After suspected bleeding, he or a family member promptly injected 1 vial of FEIBA (250–400 units corresponding to 5–10 units/kg body weight), followed by the injection of 2–10 ml saline into the needle in order t prevent local venous thrombosis. In no instance would he receive more than two injections per 24 h. Injections of FEIBA were repeated daily until subjective symptoms subsided. A physician was to be contacted as soon as possible in order to decide about supportive care, immobilisation and laboratory controls. The patient and/or family member were instructed to keep meticulous protocol, especially concerning the time of immobilisation due to haemorrhage.

Statistical Evaluation

Duration of bleeding was expressed as number of days, starting from the initial event (bleeding?) until complete mobilisation was possible. Furthermore, we attempted to grade the severity of bleeding according to the following criteria: grade 1, subjective symptoms only: grade 2: haemorrhage objectively recorded, no significant functional impairment, grade 3: objective signs of bleeding plus functional impairment (bed rest), and grade 4 major bleeding, particularly into the iliopsoas region.

Relevant episodes were recorded from the instance when an inhibitor against factor VIII prohibited further effecti treatment with factor VIII. Peak inhibitor titres ranged from 6 to 60 units/ml. Phase I includes all bleeding episodes, with and without administration of factor VIII concentrate, until therapy was switched to fraction R or FEIBA. Phase II began with the day when the patient received his first dose of fraction R or FEIBA and was instructed for home therapy For comparison of phases I and II, Wilcoxon's matched pairs signed rank test was applied.

Results

In phase I, a total of 38 haemorrhages were recorded for all patients. However of these only 26 episodes were sufficiently de-

Table I. Localisation of haemorrhage and number of bleeding episodes in 4 haemophiliacs with inhibitor against factor VIII before (phase I) and after (phase II) treatment with FEIBA

Patient	Joint bleeding				Intramuscular bleeding
	knee	ankle	elbow	shoulder	
Phase I					
A	5	–	–	–	–
B	2	1	1	–	3
C	5	1	2	–	4
D	2	–	–	–	–
Total	14	2	3	0	7
Phase II					
A	1	–	5	4	3
B	2	3	–	–	1
C	2	–	3	–	1
D	6	–	–	–	3
Total	11	3	8	4	8

fined by the protocol for statistical evaluation. In phase II, obviously due to improved instruction, 34 out of 39 haemorrhages were sufficiently defined for statistical analysis. A further impediment for objective evaluation was the fact that three episodes of bleeding into the iliopsoas region occurred during phase I only Despite these limitations it was interesting to note that bleeding sites and numbers of haemorrhages were roughly comparable in phases I or II, with about 75% of joint bleeding in both groups (table I). These same haemorrhages were graded according to their clinical severity (table II) There was no significant difference ($p > 0.1$) between the two types of treatment according to these criteria, i.e. FEIBA did not affect the types and severity of bleeding episodes. Striking differences were, however found by comparison of the duration of immobilisation (table III) The difference between the two types of treatment was highly significant ($p < 0.001$) It is apparent from the numbers of days shown in table III that the three episodes of psoas bleeding alone could not account for the difference of duration of immobilisation.

Possible side-effects attributable to FEIBA were carefully noted. 1 patient had fever immediately after injection. Tachycardia and nausea were noted once. In 1 patient, hepatitis with a positive reaction for HB-S antigen was recorded during phase II

The inhibitor titre fell in all 4 patients within weeks or months following the start of phase II. In a 5th patient with no detectable antibody after 2 years of FEIBA treatment alone factor VIII was given for treatment of a major haemorrhage into the right shoulder (not included in the study). The inhibitor promptly reappeared and precluded immediate further therapy with factor VIII

Table II. Severity of bleeding episodes listed in table I

Patient	Joint bleeding				Intramuscular bleeding
	knee	ankle	elbow	shoulder	
Phase I					
A	3,3 3,3 3	–	–	–	–
B	3,2	2	3	–	2,2,2
C	3,3,3,3 3	3	3,3	–	2,4* 4 4*
D	3,2		–		–
Phase II					
A	3	–	3,3,3 3,2	3 3,3,1	1 2,2
B	3 3	3,2,2			2
C	3,3	–	3,3,3		2,2,2
D	3,3,3,3,3 3		–		2,2,2

The numbers refer to grades of symptoms 1–4.
Iliopsoas bleeding.

Table III. Duration (days) of bleeding episodes listed in table I

Patient	Joint bleeding				Intramuscular bleeding
	knee	ankle	elbow	shoulder	
Phase I					
A	27 13,9 46,48	–			–
B	22,5	6	7	–	3 1 2
C	38,16,9 13 24	8	8,9		2,26* 43* 40*
D	10,10			–	–
Phase II					
A	3		4,3 2,5 3	3,5 5,3	2,3 2
B	6,3	3 4 4			1
C	2,5	–	2,5,3		2
D	7 5,3,2,4,4				3,–,2

Iliopsoas bleeding.

Discussion

Objective information on the therapeutic effectiveness of blood products, other than factor VIII, for treatment of haemophilia is still scanty Thus far the use of these products has been limited to patients with resistant haemophilia [6] Studies on alternative blood products are of general interest for treatment of haemophiliacs since factor VIII itself generates antibody formation in a significant proportion of treated patients,

Table I. *Localisation of haemorrhage and number of bleeding episodes in 4 haemophiliacs with inhibitor against factor VIII before (phase I) and after (phase II) treatment with FEIBA*

Patient	Joint bleeding				Intramuscular bleeding
	knee	ankle	elbow	shoulder	–
Phase I					
A	5	–	–	–	–
B	[illegible]	1	1	–	3
C	5	1	2	–	4
D	2	–	–	–	–
Total	14	2	3	0	7
Phase II					
A	1	–	5	4	3
B	2	3	–	–	1
C	2	–	3	–	1
D	6	–	–	–	3
Total	11	3	8	4	8

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In any event, we would strictly warn against allowing a given patient to administer FEIBA for more than a few days without proper laboratory monitoring, particularly measurement of fibrinogen and platelet counts.

Our study has the character of a preliminary clinical investigation which indicates mainly that low-dose FEIBA therapy outside the hospital environment is feasible. Whether or not this treatment is truly justified and superior to any other proposed forms of treatment remains to be shown by prospective, randomized and controlled studies.

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E. A. Beck, MD,
Central Haematology Laboratory
Inselspital, CH 3010 Bern (Switzerland)

i.e., about 10% or more of patients with severe haemophilia can no longer profit from the benefit of classical replacement therapy [1 4 6 10] In theory bypassing of factor VIII, e.g., by activated factor X, is possible. Thus a number of activated blood products have been tested in patients who were resistant to conventional therapy [5 6 9] As pointed out by *Bloom* [6] no firm conclusions about the safety and efficacy of this approach can be drawn from the available literature.

The mode of action of FEIBA on the coagulation pathway *in vitro* and a possible haemostatic effect *in vivo* remain a matter of speculation [6 7] It is already apparent from the available literature [2, 6, 11 12] that FEIBA, despite its improved standardisation in comparison with other activated products, is probably not equivalent to the haemostatic effect of factor VIII in uncomplicated haemophilia. Dosage of FEIBA in resistant haemophilia seems to be limited by its capacity to induce intravascular coagulation [14]

When we started to use FEIBA and its predecessor fraction R, for treatment of haemophiliacs, we were fully aware that no firm basis existed for the application of this product. However the clinical situation of haemophiliacs who were to receive this experimental product was grave if not desperate in that conventional treatment with factor VIII and/or supportive care gave highly unsatisfactory results thus, prolonged periods of bed rest were inevitable. The first problem which we encountered was the lack of a clearly established dose-response relationship Our practical approach, including later dose regimens for home treatment, was based on the assumption that, above a critical step of activation, there might be an all-or-nothing effect *in vivo* Therefore, we did not adjust the dosage to the severity of bleeding, nor to the amount of circulating inhibitor Furthermore, in view of the possibility of intravascular coagulation, dosage of FEIBA was significantly lower than that proposed by the producer Thus, the daily dose did not exceed 800 units, even in adults.

While FEIBA was clinically well tolerated by the majority of our patients we still encountered two serious side-effects which were probably caused by this product, i.e., one episode of hepatitis and one severe hypersensitivity reaction in 1 patient who had not tolerated other blood products as well. Other recorded side-effects, such as tachycardia, fever and nausea, were infrequent and lack specificity At the low doses of FEIBA, used in this study the inhibitor titre decreased or disappeared in all patients.

Our attempt to test the effectiveness of FEIBA by comparing the duration of bleeding before and after giving this product strongly suggests that morbidity is reduced by immediate FEIBA treatment of suspected bleeding. In all 4 patients who had received FEIBA, comparable types of bleeding were clearly causing less immobilization than bleeding treated with factor VIII and/or supportive care alone. Our results suggest that FEIBA is particularly effective in shortening the morbidity caused by joint or minor muscular bleeding. In 1 patient FEIBA failed, however to stop bleeding following tooth extraction, a situation which could be easily controlled by factor VIII administration despite the presence of an inhibitor Our study also indicates that home therapy using FEIBA, is feasible, at least in patients who tolerate the product. Special precautions are needed to prevent thrombosis of blood vessels at the site of injections.

be more carefully scrutinized. We have reviewed 124 cases of patients with a diagnosis of A.A. seen in our service between 1971 and 1978, in order to evaluate the degree of effectiveness of treatment with androgens and the prognostic factors related to a favorable or unfavorable response.

Material and Methods

Of the 124 A.A. cases, 58 patients were male and 66 female; the mean age was 47.5 years (range 16 to 76), and the average duration before treatment with androgens was of 9.08 months (range 0.3 to 72 months).

The diagnosis of A.A. was based on Wintrobe's criteria [32]: pancytopenia as evidenced by diminished production of all the blood elements formed in the bone marrow including hypoplasia or severe aplasia of the bone marrow with no indication of primary infiltrative disease, replacement or suppression of active hematopoietic tissue.

The clinical history of all the patients was compiled, with complete blood count, serum iron, vitamin B12 and serum folate, Ham's and sucrose hemolysis tests, uric acid, blood sugar, BUN, cholesterol, bilirubin, serum protein electrophoresis, transaminases, alkaline phosphatase and lactic dehydrogenase. Complete urine analysis was made. Hepatosplenic scintigrams and bone marrow aspirate and biopsy were also done.

The patients were divided into severe (54) and mild (70) cases, following the Seattle Group criteria [27]. A case was classified as severe if the patient had hypoplasic bone marrow and 2 out of 3 of the following parameters in the two first blood counts: (1) platelet count less than 20,000/cumm, (2) neutrophile count less than 500/cumm, and (3) corrected reticulocyte count less than 1 % in the presence of anaemia. Otherwise, the case was classified as mild.

The signs and symptoms in these two groups were similar but the frequency of infection and transfusion requirements were higher in the severe cases. In 58 patients (46.9%) there was no contact with toxic substances or drugs.

In the remainder the A.A. was considered secondary to one of the following substances: oxyphenbutazone, chloramphenicol, benzene, DDT and/or hair dye. In each of these latter cases there was temporal relationship between the first appearance of symptoms and contact or previous contact with the substance (tables I and II).

The treatments used were oxymethalone (17β-hydroxy-17α-methyl-2-hydroxymethylene 5α-androstan-3-one), 1-3 mg/kg/day for periods of 3 months or more, methalone (17β-hydroxy-2α-methyl-5α-androstan-3-one), 5-8 mg/kg/week intramuscularly for periods of 3 months or more and prednisone in diminishing doses beginning with 150 mg/day for 5 days until 2,100 mg had been administered.

Some patients received different treatment if the first failed, thus methalone and cyclophosphamide were used in patients who did not re-

Table I. Aetiology of aplastic anaemia in 124 patients

Agent	No. patients
Idiopathic	58
Oxyphenbutazone	20
Cloramphenicol	3
Benzene	20
DDT	20
Hair Dye	3
	124

Table II. Response in relation to aetiology and severity in 60 patients with aplastic anaemia

Agent	Severe		Mild	
	No.	Response	No.	Response
Idiopathic	3	0	19	13
Oxyphenbutazone	1	0	6	2
Chloramphenicol	1	1	1	1
Benzene	5	0	9	8
DDT	2	0	11	6
Hair Dye	1	0	1	0
Totals	13	1	47	30

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Use of Androgens in Acquired Aplastic Anaemia

Relation of Response to Aetiology and Severity

Javier Pizzuto Guillermo Conte Armando Sinco Manuel Morales Agustin Avilés Raul Ambriz and Américo Fernández

Hematology Service, General Hospital of the National Medical Center I.M.S.S. Mexico City Mexico

Key Words. Aplastic anaemia aetiology Prognostic indices androgens severity

Abstract. 124 adult patients with acquired aplastic anaemia (A.A.) were studied. 54 presented as severe A.A. (mean survival of the whole group 2.7 months and in those who died 2.5 months mortality 88.8%) while 70 were mild (mean survival of the whole group, 27.2 months and in those who died, 22.7 months mortality 28.5%). Sixty four did not live long enough to have adequate therapeutic trials or developed severe hepatic failure which made it impossible to continue treatment. The effectiveness of 78 therapeutic periods of treatment in the 60 evaluable cases was analyzed as to aetiology and severity A response was obtained in 31 (40%) of which 24 were with oxymethalone 17 showed improvement after 6 months of treatment. No conclusions could be drawn as to the effectiveness of methalone, cyclophosphamide and prednisone because of the limited number of treatments. In severe A A. only one of 13 treatments was effective, whereas 30 of the 65 used in mild A.A gave a response Oxymethalone was used in 23 out of 41 (56%) The aetiology sex and age appeared to have no influence on the response of the 60 patients analyzed. The only factor which appears useful for prognosis as to the effectiveness of treatment is the severity of the case.

Introduction

Androgen therapy of aplastic anaemia (A.A.) was introduced almost two decades ago, and appeared to offer much promise for improving the grave prognosis of this disease [22] Subsequent experience with androgen therapy of A.A. has been somewhat disappointing [6 30 31] The discrepancy between initial results and later experience has not yet been explained as due either to ineffectiveness of the androgens or to aetiological factors such as drug or toxic substances. A retrospective study was undertaken to attempt to determine variables, such as severity of A.A., mode of therapy etc., which had favorable or unfavorable prognostic implication and which could serve as the basis for a subsequent prospective controlled study in which such variables could

Table III. Response to treatment in 60 A.A. patients (78 therapeutic periods)

	Response in relation to severity				Duration of treatment (in those responding) No. of Treatments		Total studies	
	Severe		Mild					
	No. of Treatments	Response	No. of Treatments	Response	3 mo.	6 mo.	Number	Response
Oxymetholone	10	1	41	23 (56%)	7	17	51	24 (47%)
Methalone	3	0	8	3	3	0	11	3 (27%)
Cyclophosphamide	0	0	7	3	3	0	7	3 (43%)
Prednisone	0	0	9	1 (11%)	1	0	9	1 (11%)
Totals	13	1 (8%)	65	30 (46%)	14	17	78	31 (40%)

Discussion

The treatment of A.A., has successively passed from symptomatic measures (transfusion and other supportive therapy) to a trial of splenectomy and then to the use of adrenocortical steroids, but none of these modes of therapy appears to have had a favorable effect on prognosis. In the last two decades the use of androgens appeared promising, a high percentage of responses being reported [22] However other authors have found a much lower proportion of re sponse to these drugs, and the present study was undertaken to investigate this diver gence of opinion and the real usefulness of the treatment with androgens. Other treat ments for A.A. have been reported recently bone marrow transplant and immune-suppressive therapy oriented to correct the most important pathogenic mechanisms of this condition [5 11] cell stem defect (qual itative or quantitative) or possible autoimmune changes in the microenvironment.

Bone marrow transplant has given promising results [26], as has the use of immunosuppressors such as cyclophosphamide [4 19] or antilymphocyte globulin [12, 25]

An analysis of our 124 patients indicates that although the group included a high per centage of secondary A.A. (53 1 /), there was no essential difference in response rate or course between these and the idiopathic cases. Whatever the sex, age and aetiology of the A.A., almost the only index for prognosis is the gravity of the case. It may be mentioned, however that of the 13 treat ments used in severe cases (table II) only 1 of the 2 exposed to drugs responded and none of the idiopathic cases or those ex posed to toxic substances gave any re sponse

An overall analysis of our 60 evaluable patients shows that a substantial proportion of cases responded to androgen therapy (43.5), as has been observed by some other authors. However if the cases are classified according to the severity of the disease,

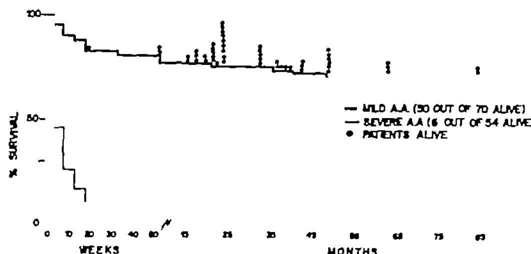


Fig. 1. Kaplan-Meier [16] product limit estimates percentage surviving for 74 patients with mild aplastic anaemia and for 50 with severe aplastic anaemia ($p < 0.0001$).

spond to either oxymethalone or prednisone. We have consequently analyzed the response to each type of treatment rather than the response of the individual patient.

Sixty-eight of the 124 patients died, and of these, 48 belonged to the severe A.A. group which had a mean survival of 2.5 months in those who died (range 0.06 to 20 months) and an 88.8% mortality. The remaining 20 deaths belonged to the mild A.A. cases which had a mean survival several times greater than the preceeding group (22.4 months in those who died, range 0.6 to 84 months) and a mortality only 1/3 that of the severe cases (28.5%) (fig. 1).

Of the entire group of 124 patients, 64 were therapeutically unevaluable because the treatment could not be administered over a 3 month period. 45 patients (35 severe and 10 mild) died shortly after diagnosis, and 19 (6 severe and 13 mild) developed severe liver failure. It is interesting to note that in this last group one of the patients had a spontaneous remission. The therapeutic analysis of this study was, therefore, carried out on only 60 evaluable patients.

An important number of the 60 patients did not respond to any of the treatments employed. Of the 78 therapeutic periods analyzed there was a response in only 31 (40%). Of the 51 cases (10 severe, 41 mild) which received oxymethalone, 24 responded (47%), of 11 (3 severe and 8 mild) that received methalone, only 3 (27%) responded, and of two groups of 7 and 9 each (mild), treated respectively with cyclophosphamide and prednisone, there was a response in 3 patients in the former (43%) and only one (11%) in the latter (table III).

Most cases that responded to oxymethalone did so after 6 months of treatment, while in those treated with methalone, cyclophosphamide or prednisone, the response, if it appeared, did so within the first 3 months after the beginning of the treatment (table III). In 22 cases the response to oxymethalone was partial (normalization of haemoglobin and haematocrit), and complete in only 2 (return to normal red cell, white cell and platelet count). with methalone, cyclophosphamide and prednisone the responses were partial. Of all the responding patients only two that received oxymethalone suffered a relapse, without apparent cause, and in one other there was a response that depended on continued administration of oxymethalone.

An analysis of the above results reveals an almost complete lack of response in severe cases of A.A., only one of the 10 cases treated with oxymethalone responded. In mild cases the frequency of partial or complete response to treatment varied from 11% to 56% with oxymethalone giving the largest number of responses (table III).

An analysis of the response to treatment indicates no correlation between sex, age, aetiology and response, but only between the degree of severity of the condition and the response (table II).

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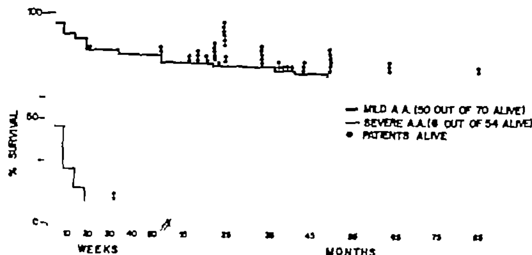


Fig. 1. Kaplan Meier [16] *product limit estimates* percentage surviving for 74 patients with *mild aplastic anaemia and for 50 with severe* aplastic anaemia ($p < 0.0001$).

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The satisfactory response to oxymethalone achieved in this study is mainly observed in the cases in which it was administered for a long time: 70% of the responding cases received this androgen for at least six months (table III). It is not possible to determine whether there was spontaneous response in any of them, except that in one case the response depended on the continued use of oxymethalone.

If oxymethalone requires this prolonged use, it cannot be expected to be very effective with severe cases, which have such a brief survival time (mean survival of 2.7 months in 54 patients and in those who died 2.5 months fig. 1) and high mortality rate (88.8%). This is borne out in our results of 10 severe cases that received it, only one responded (table III).

The difference of opinion as to the effectiveness of androgens may derive from the grouping together of A.A. cases of all degrees of severity Thus, a review of the literature [1–3 6–10 13–15 17–18, 20–24 28–31] and the present publication yields 903 cases of A.A. An analysis of the response to different therapeutic trials without taking into consideration the severity of the A.A. gave an average of 41 7%. However when the authors data permitted an analysis in terms of the severity of the case (table IV), a different frequency of response is evident mild cases frequently responded to treatment while groups with a greater number of severe cases, with their high mortality and short survival-time gave unsatisfactory results.

We may conclude that patients with severe A.A. are candidates for a different type of therapy such as bone marrow transplant if a compatible donor is available [26, 27] or immunosuppressive treatment with cyclophosphamide [4 19] or antilymphocyte globulin [12, 25]

Acknowledgement

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Table IV Response in relation to severity of aplastic anaemia

Author	Treatment	Unclassified		Classified by Present Authors from Data in Original Papers.			
				Severe		Mild	
		Cases	Response, %	Cases	Response, %	Cases	Response, %
Shahidi and Diamond [22] 1961	Testosterone	17	53%	14	42%	3	100%
Dorantes et al. [10] 1965	Testosterone, oxymethalone ACTH	54	44%	25	12%	29	72%
Alvarez et al. [3] 1969	Testosterone, Androstenalone	32	44%	12	25%	20	55%
Davis and Rubin [9] 1972	Nandralone	24	21%	17	6%	7	57%
Alexanian et al. [1] 1972	Oxymethalone	6	0%	–	–	6	0%
Camitta et al. [7] 1976	Oxymethalone Methalone Nothing	17 7 7					
		31	19%	31	19%		
Branda et al. [6] 1977	Nandralone	8	25%	4	0%	4	50%
Van Hengstum et al. [29] 1979	Oxymethalone Methenalone	3 19					
		22	50%	14	50%	8	50%
Pizzuto et al. (Present study)	Oxymethalone Methalone	51 11	47% 27%	10 3	10% 0%	41 8	56% 37%
Totals		356	38.2%	130	20.7%	126	56.3%

a different picture emerges there is a much higher likelihood of response to androgens, and indeed to the other drugs used, in mild cases of A.A., but in severe cases the likelihood of response is minimal (about 8%) Adrenocortical steroids appear not to be very useful. The usefulness of cyclophosphamide could not be evaluated because of the limited number of patients and the fact that the instances of successful use were in mild A.A. where the percentage of response was similar to that with the other treatments there even existed the possibility of spontaneous response.

Nevertheless, the response in 3 out of 7 cases previously treated unsuccessfully with oxymethalone and prednisone suggests that cyclophosphamide may have some value.

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Hematological Abnormalities in Scleroderma

A Study of 180 Cases

Rida A. Frayha, Lawerence E. Shulman and Mary B. Stevens

Connective Tissue Division, Department of Internal Medicine, The Johns Hopkins School of Medicine and the Department of Rheumatology, Good Samaritan Hospital and the Johns Hopkins Hospital, Baltimore, Md., and Department of Medicine, American University Medical Center, Beirut

Key Words. Hematological abnormalities · Scleroderma

Abstract. Hematological abnormalities in scleroderma indicate a specific complication of the disease itself, or an associated illness. Among 180 patients with scleroderma, anemia was detected in 25% and was attributed to chronic inflammatory disease (usually an overlap syndrome), bleeding mucosal telangiectases as part of the CREST syndrome, intestinal malabsorption, and microangiopathic hemolysis. Leukocytosis, present in 14%, was correlated with active myopathy and/or advanced visceral involvement while leukopenia was suggestive of a crossover with systemic lupus erythematosus. Thrombocytopenia was often a manifestation of microangiopathy and thrombocytosis was associated with an arteritis or a tumor syndrome.

Systemic sclerosis (scleroderma), in contrast to systemic lupus erythematosus, is characterized by a paucity of hematological abnormalities. As part of a comprehensive analysis of 180 patients with scleroderma, we were impressed that when present, these abnormalities suggest either a specific complication of the ailment itself, or a concomitant disease process. This has prompted us to report herein on the frequencies, etiologies, and significance of the hematological derrangements in scleroderma.

Materials and Methods

The case records of all patients with scleroderma hospitalized at the Johns Hopkins Hospital from January 1952 to December 1972 were reviewed. The criteria for the diagnosis of scleroderma were as proposed by *Medsger and Masi* [13]. 180 patients, 131 females and 49 males, satisfied these criteria. The subset of scleroderma designated as the CREST syndrome was considered when the predominant features in patient with scleroderma were calcinosis cutis, Raynaud' phenomenon, esophageal hypomotility, sclerodactyly and telangiectases [7]. Patients with concomitant systemic lupus erythematosus (SLE) or rheumatoid arthritis were classified according to the relevant criteria of the American Rheumatism Association [3, 5]. The criteria of *Bloch et al.* [1] were used for the diagnosis of Sjögren's syndrome.

Anemia was defined in the female cases as a hematocrit below 37% or a hemoglobin concentration less than 12 g/100 ml and in the male cases, as hematocrit below 40% or hemoglobin less than 13 g/100 ml. The normal range of the white blood cell (WBC) and platelet counts were consid-

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Javier Pizzuto, Hematology Service,
General Hospital of the National Medical Center
IMSS Mexico City Mexico

In 4 patients, the anemia resulted from gastrointestinal bleeding: in 1 secondary to peptic ulcer and in another secondary to uremia. In 2, the etiology of the bleeding was not found.

Renal failure was considered to be the cause of the anemia in 3 patients: 1 had scleroderma kidney and microangiopathic anemia and the other 2 had pyelonephritis. 1 patient became anemic with the onset of lymphosarcoma. In 3 patients the etiology of the anemia remained obscure.

White Blood Cells

In 25 patients (14%) the WBC was elevated (range = 10,200–24,500/mm³) in the absence of a recognized infection or steroid therapy. Of these, 18 had normal differential counts, 5 had neutrophilia, and 2 had eosinophilia (highest = 770/mm³). 10 of the 25 patients had active polymyositis. However, there was no statistically significant difference in leukocytosis among our scleroderma patients with and without polymyositis (p greater than 0.05). 6 patients had marked visceral involvement with advanced pulmonary scleroderma disease in 4 and intestinal malabsorption in 2. Scleroderma kidney was diagnosed in 2 patients and another 2 had active rheumatoid arthritis. In the remaining 5 patients with leukocytosis, there was no advanced visceral involvement and no cause for the leukocytosis was obtained.

10 patients (5.5%) had repeated WBC counts between 3,200 and 4,999/mm³. Of the 4 patients with counts between 3,200 and 3,999/mm³ 3 had SLE (with concomitant Sjögren's syndrome in 1) and lupoid hepatitis was diagnosed in the 4th. Among the 6 patients with counts between 4,000 and 4,999/mm³ only 1 had SLE. There was no evidence of any concurrent disease process in the other 5.

Platelets

The platelets were evaluated by peripheral blood smear study in all 180 patients and by platelet count in 86. 8 patients had thrombocytopenia on more than one occa-

Table II. Derrangement in white blood cell and platelet counts

Etiology	Leukocytosis (n = 25)	Leukopenia (n = 10)	Thrombocytopenia (n = 8)	Thrombocytosis (n = 4)
Overlap syndrome				
SLE		5[1]		
Polymyositis	10			
Rheumatoid arthritis	2			1
Sjögren's syndrome				1
Scleroderma kidney[3]	2		4	
Advanced scleroderma	6			
Malignancy				1
Unknown	5	5	2	1

1 patient had lupoid hepatitis, another had Sjögren's syndrome.
Thrombocytosis attributable to an arteritis.
Associated with microangiopathic hemolytic anemia.

ered as 5 000–10 000/mm³ and 100,000–400 000/mm³ respectively. The diagnosis of iron deficiency anemia was made according to peripheral blood smear, serum iron, total iron-binding capacity and marrow iron store evaluation. Vitamin B_{12} absorption was assessed by the Schilling test. Standard criteria were employed for the diagnosis of microangiopathic hemolytic anemia [17] and the anemia of chronic disease [2]. Intestinal malabsorption was considered to be present if the classical clinicoradiological features were present along with a fecal fat excretion of more than 6 g/24 h, with or without a low xylose excretion or a low serum carotene concentration.

Results

Significant Anemia (table I)

19 patients (10.6%) had a hematocrit of 33% or less. Of these, 7 had renal failure which was due to scleroderma kidney in 6. All 6 had microangiopathic hemolytic anemia as a preterminal event. The seventh patient had nephrosclerosis and pyelonephritis.

Second to renal failure ranked the CREST syndrome [7]. All of 6 such patients had iron deficiency anemia, attributed to gastrointestinal bleeding in 5. 4 of the 6 subjects had mucosal telangiectases (in the esophagus in 1, stomach in 1, and rectum in 2). Blood transfusions were given to 1 of these patients and in all the anemia was corrected with oral iron therapy. 2 patients – 1 having the CREST syndrome – had intestinal malabsorption and iron deficiency anemia.

4 had features of other connective tissue diseases: 1 rheumatoid arthritis, 1 polymyositis, 1 discoid lupus, and 1 SLE (without hemolysis). The anemia was considered secondary to the associated chronic inflammatory disease. None had malignancy. In 1 patient the etiology of the anemia was not found.

Moderate Anemia

26 patients (14.4%) had moderate anemia (hematocrit 34–36% for females, and 34–39% for males). 14 had features of one or more connective tissue disease(s): 12 with polymyositis, 4 with Sjögren's syndrome, 1 with rheumatoid arthritis, 2 with discoid lupus, and 1 with SLE. Of these, 4 had iron deficiency anemia, and the 1 with SLE had Coombs' positive hemolytic anemia. The anemia in the rest was attributed to the chronic inflammatory process.

6 patients (4 with associated polymyositis) had intestinal malabsorption and iron deficiency anemia. One, in addition, had folic acid deficiency. All had extensive small bowel disease and all had hypoalbuminemia, hypocholesterolemia, and hypocalcemia with tetany in 2. In 3 patients in whom the Schilling test was done, B_{12} absorption was normal. In 1 patient studied by ^{51}Cr labeled albumin, protein losing enteropathy was not found. All had biochemical and clinical remission after long-term tetracycline therapy and iron replacement.

Table I. Etiologies of anemia in scleroderma

	Significant anemia (n = 19)		Moderate anemia (n = 26)	
Uremia	7		3	
MAHA		6		1
CREST syndrome	6			
GI bleeding		5	4	
Malabsorption	2		6	
Overlap syndrome	4		14	
Malignancy	0		1	
Unknown	1		3	

1 Some patients had more than one etiology.
2 Microangiopathic hemolytic anemia.

In 4 patients, the anemia resulted from gastrointestinal bleeding· in 1 secondary to peptic ulcer and in another secondary to uremia. In 2, the etiology of the bleeding was not found.

Renal failure was considered to be the cause of the anemia in 3 patients, 1 had scleroderma kidney and microangiopathic anemia and the other 2 had pyelonephritis. 1 patient became anemic with the onset of lymphosarcoma. In 3 patients the etiology of the anemia remained obscure.

White Blood Cells

In 25 patients (14 /), the WBC was elevated (range = 10,200–24,500/mm³) in the absence of a recognized infection or steroid therapy Of these, 18 had normal differential counts, 5 had neutrophilia, and 2 had eosinophilia (highest = 770/mm³). 10 of the 25 patients had active polymyositis. However there was no statistically significant difference in leukocytosis among our scleroderma patients with and without poly myositis (p greater than 0 05). 6 patients had marked visceral involvement with advanced pulmonary scleroderma disease in 4 and intestinal malabsorption in 2. Scleroderma kidney was diagnosed in 2 patients and another 2 had active rheumatoid arthritis. In the remaining 5 patients with leukocytosis, there was no advanced visceral involvement and no cause for the leukocytosis was obtained.

10 patients (5.5°/) had repeated WBC counts between 3,200 and 4,999/mm³ Of the 4 patients with counts between 3,200 and 3,999/mm³ 3 had SLE (with concomitant Sjögren s syndrome in 1) and lupoid hepatitis was diagnosed in the 4th. Among the 6 patients with counts between 4 000 and 4,999/mm³ only 1 had SLE. There was no evidence of any concurrent disease process in the other 5

Platelets

The platelets were evaluated by peripheral blood smear study in all 180 patients and by platelet count in 86. 8 patients had thrombocytopenia on more than one occa-

Table II. Derrangement in withe blood cell and platelet counts

Etiology	Leukocytosis (= 25)	Leukopenia (= 10)	Thrombocytopenia (n = 8)	Thrombocytosis (n = 4)
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SLE		5	2	
Polymyositis	10			
Rheumatoid arthritis	2			1
Sjögren's syndrome				1
Scleroderma kidney	2		4	
Advanced scleroderma	6			
Malignancy				1
Unknown	5	5	2	1

1 patient had lupoid hepatitis, another had Sjögren's syndrome.
Thrombocytosis attributable to an arteritis.
Associated with microangiopathic hemolytic anemia.

sion the lowest count being 30 000/mm³ Bone marrow studies revealed a normal or increased number of megakaryocytes. 6 of these patients died 4 of malignant hypertension and azotemia. All 4 had microangiopathic hemolytic anemia 1 died secondary to an overlap syndrome with features of scleroderma polyarteritis nodosa and SLE. The 6th patient succumbed to refractory lupoid hepatitis. In all the thrombocytopenia was a preterminal finding. In the other 2 patients no etiology of the thrombocytopenia was found 1 is living and the other is lost to follow-up None had sepsis or hypersplenism, and in none were drugs incriminated. Bleeding manifestations were not encountered in any of our thrombocytopenic patients.

4 patients had persistent thrombocytosis. In 2, it was attributed to a vasculopathy in 1 due to rheumatoid arthritis and arteritis, and in the other due to Sjögren s syndrome. In the 3rd patient, the thrombocytosis was related to a tumor syndrome (lymphosarcoma) No explanation of the thrombocytosis was available in the 4th. All but the 1 with Sjögren s syndrome, died shortly after the detection of thrombocytosis.

Discussion

In their report of 150 patients with scleroderma, *Leinward et al.* [12] found no anemia in the 'uncomplicated' cases. The frequency of anemia, however in two more recent studies of scleroderma ranged between 7 and 29% [19 20] In our series, 25% of the patients developed anemia. The present data confirm that of *Westerman et al.* [20] in that iron deficiency is the underlying etiology of the anemia in an appreciable number of patients half of theirs, and one third of ours

The commonest cause of the iron deficiency anemia in our patients was gastrointestinal bleeding, often from mucosal telangiectases In our experience the association of anemia and such telangiectases in scleroderma suggests gastrointestinal bleeding and that endoscopy might be helpful in identifying these telangiectases. In some patients intestinal malabsorption contributed greatly to the iron deficiency anemia. The reported B_{12} malabsorption in scleroderma [20] was not observed in 3 of our patients thus studied. The malabsorption in our patients proved to be tetracycline responsive. Of interest is the recently described megaloblastic anemia following long-term tetracycline therapy for acne [11] Although this has not been yet appreciated, such a potential complication should be considered during the management of the malabsorption of scleroderma.

In some of our patients no specific etiology of the iron deficiency anemia could be found. Two-thirds of our anemic patients were females. Anemia secondary to menstrual blood loss stands as a possibility Also, it is tempting to speculate that intermittant occult bleeding from ulcerations along the alimentary tract, known to occur in 40% of scleroderma patients [4] might have resulted in anemia.

In some reported cases with the CREST syndrome and primary biliary cirrhosis, bleeding esophageal varices contributed, in part, to the anemia [1] However of our 180 patients with scleroderma, the only one with both of these diseases had neither esophageal bleeding nor anemia. This, coupled with the relatively low incidence of cirrhosis in scleroderma [4] indicates that anemia secondary to bleeding varices is infrequent.

Of interest is that 40% of our anemic patients with scleroderma had other connective tissue diseases. 5 resembled the newly described entity of mixed connective tissue disease [18]. However the extractable nuclear antigen (ENA), characteristic of this syndrome, was not sought. About half of the described patients with this syndrome are anemics. Furthermore, 2 of our patients had scleroderma and SLE both resembling the syndrome of scleroderma with serologic and systemic manifestations of SLE reported by *Dubois et al.* [6]. Two-thirds of their patients had significant anemia. In our patients with an overlap syndrome, the anemia was compatible with that of chronic disease. Besides, 1 patient with SLE had autoimmune hemolytic anemia. Coombs' positive hemolytic anemia had been described previously in 4 patients with scleroderma [8–10, 16, 20] and in 4 others with scleroderma as part of an overlap syndrome [6, 18].

The total and differential white blood cell counts are usually normal in scleroderma. Of our 180 patients with scleroderma, 43 had polymyositis. Of these scleropolymyositics, 10 had leukocytosis, with neutrophilia in some, in the absence of a recognized infection or steroid therapy. Likewise, neutrophilic leukocytosis has been encountered in some cases of acute idiopathic polymyositis [14]. In our patients, overall, the leukocytosis was correlated with advanced scleroderma and visceral involvement.

Leukopenia in scleroderma is of discriminative value, and as illustrated by our patients, its presence strongly suggests an overlap syndrome with SLE. In three large series, none of over 1,000 patients with scleroderma had leukopenia [12, 19, 20]. On the other hand, 6 of 11 (55%) of patients with scleroderma and SLE had low WBC [6]. A similar incidence of leukopenia was found in patients with the mixed connective tissue disease [18].

Our data suggest that when thrombocytopenia develops during the course of scleroderma a microangiopathic syndrome related to scleroderma kidney or less commonly a crossover with SLE should be considered. In 2 of our patients, no cause of the thrombocytopenia was found, and whether these resemble the recently described cases of scleroderma and autoimmune thrombocytopenia [9] remains speculative. Likewise, thrombocytosis indicates a serious concurrent disease such as arteritis or tumor. Hence, deviation in platelet counts are, on the whole, grave prognostic signs.

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Dr R. A. Frayha, Department of Medicine, American University Medical Center Beirut (Lebanon)

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Serological and Immunoglobulin Studies in Autoimmune Haemolytic Anaemia with Emphasis on the Nature of Biphasic Antibodies

S. R. Joshi, Y. S. Iyer and H. M. Bhatia

Blood Group Reference Centre (ICMR), Seth G.S. Medical College, Bombay

Key Words. Autoantibodies · Immunoglobulin · Serology

Abstract. 55 cases of autoimmune haemolytic anaemia with positive direct Coombs test were investigated for the nature of autoantibodies. The studies involved characterization of the autoantibodies on the basis of their thermal amplitude, serological specificity, nature of immunoglobulin and light chain specificity. The findings showed that the autoantibodies could be classified as cold autoantibody (18 cases), warm autoantibody (5 cases) and biphasic autoantibody which reacted at 37 °C as well as at 6 °C (32 cases). Further classification of these cases was made on the basis of antibody specificity to blood groups, immunoglobulin type in relation to the optimum temperature of their reaction.

Introduction

Autoantibodies associated with acquired haemolytic anaemia are classified as 'cold' and 'warm' autoantibodies on the basis of their thermal amplitude [13]. Serologically, autoantibodies show a specificity to some of the common blood group antigens. Those reacting at cold temperatures show the specificity within the I-i system – often directed towards the antigenic complex of the I-i and the ABO system. Occasionally, the cold autoantibodies have also been reported as anti-P, anti-N and anti-Pr [8]. 'Warm' autoantibodies have specificities commonly directed towards the antigens of the Rh system, mainly to the antigen e and occasionally to other antigens or antigenic complexes within the Rh system [8]. 'Cold' autoantibodies usually occur as IgM immunoglobulins [5] though rare cases with IgG and IgA specificities have also been reported [1]. The majority of warm autoantibodies display IgG specificity, the IgM and IgA types being rare [4]. Since most of the pathological antibodies are produced by a single abnormal immunocyte clone, the autoantibodies are either of the kappa or the lambda type [8]. Levels of immunoglobulins in patients with autoimmune haemolytic anaemia show a deviation from the normal pattern and is characteristic of the type of the disease [6].

The present study reports the nature of autoantibodies in 55 patients suffering from autoimmune haemolytic anaemia.

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37 C having Rh specificity. On the other hand saline-reacting cold antibody was present in 17 cases and 14 of them had specificities within the I-i complex. 11 cases possess low titre saline-reacting antibodies which gave equivalent reactions at 6 and 37 C (titre range 1:1–1:4).

In all these 32 cases of biphasic autoantibody, the incomplete type detected by the enzyme or the ICT technique showed a higher titre than the saline type. The comparative titre values of these incomplete antibodies at two different temperatures and their correlation with their serological specificity is given in figure 1. It is obvious that the cases having incomplete antibody titre of 1:32 or more at 37 C possess specificities predominantly within the Rh system. 14 cases with the I-i specificity were all predominantly of the cold-reacting type. Those which showed no clear-cut specificity were within the titre range of 1:8–1:16 at both temperatures.

Titre at 4 °C	Titre at 37 °C: 2	4	8	16	32	32	Total
4					●●		3
8							2
16			●●●				7
32			○●●	●●		●●	10
32		□□□□	□□□□			○	10
Total		8	1	3	4	8	32

Fig. 1. Correlation of incomplete antibody titre at 6 and 37 °C and their blood group specificities among the biphasic autoantibodies. □ = I-i specificity; ● = Rh specificity; ○ = non-specific.

Blood Group Specificity of Autoantibodies

Table III gives the finding of the serological specificity of 55 autoantibodies. Those with I specificity showed a wide spectrum of reactions. Only one serum showed loss of reactivity with enzyme-treated cells, suggesting Pr specificity. Among those showing specificity within the Rh system there were 3 cases with D-like and 3 cases having complex antibodies classified as pdl as they gave negative or weak reactions with

Table I. Immunoglobulin and light chain specificities involved in autoantibodies

Autoantibodies	n	Effect of β-mercaptoethanol: affected	partially affected	not affected	Immunoglobulin specificity: IgG	IgM	IgG+M (dual)	Light chain specificity: kappa	lambda	kappa + lambda (dual)	not tested
Auto-cold	18	18				18	–	7	2	1	8
Biphasic											
Predominantly cold	15	14	1			14	1	10	3	–	2
Predominantly warm	9		6	3	2		7	5	–	–	4
Rest	8	1	3	4		1	7	2	2		4
Auto-warm	5		2	3	4		1		1	1	3
Total	55	33	12	10	6	33	16	4	8	2	21

Materials and Methods

Blood samples of the patients were referred by various haematology clinics and local hospitals in Bombay and other parts of India. Reagents like monospecific antiglobulin sera were prepared locally

Serological specificity was determined by titration of antibodies at various temperatures, using panels of red blood cells which included cord blood and other rare phenotypes like i-I, I i- D- and Rh null. Selective absorption and elutions were also carried out in order to determine the specificity

Immunoglobulin and light chain specificity of autoantibodies was carried out by the indirect Coombs test on native sera using monospecific antiglobulin reagents and the results were confirmed by passive haemagglutination inhibition of monospecific antiglobulin reagents with the eluates prepared from autoimmune sera. Eluates from sensitized cells were prepared by heat elution techniques [9] in cases with cold autoantibodies, whereas eluates in cases with warm autoantibodies was prepared by the ether elution technique [12]

Classification of I antibodies into IT ID and IF was based on the reaction pattern reported by *Race and Sanger* [11] Among those having Rh specificity the pdl specificity was identified by negative or weak reaction with Rh null in comparison to normal and -D- cells [8] D-like and C-like antibodies were those showing preferential reactivity with the blood having the respective antigens.

Test for the Estimation of Immunoglobulin Levels

The sera to be tested for immunoglobulin level were diluted in twofold serial dilution in normal saline up to 20 tubes. Then, suitably diluted monospecific antiglobulin reagents having a titre of 1 8–1.16 were added. The mixture was kept at room temperature for 20 min. A volume of the suspension of red cells sensitized with IgG or IgM or IgA immunoglobulin was added to the test. After 5 min incubation at room temperature, the test was spinned at 1,000 rpm for 1 min. The results were read macroscopically and negative results were confirmed microscopically No agglutination indicated the inhibition of antiglobulin activity suggesting the presence the particular immunoglobulin. These results were compared with a WHO standard control serum having IgG 96.2 IU IgM 96.2 IU and IgA 95.3 IU

Results

55 autoimmune cases with positive direct Coombs test were studied for serological and immunological aspects. These include 18 cases with autoantibody reacting only at 6 C, 5 cases with autoantibody reacting only at 37 C and 32 cases of autoantibodies reacting at both 6 and 37 C (biphasic).

Cold Reacting Autoantibodies (18 cases)

All these cases had a saline reaction as well as incomplete autoantibody The titre of the antibody ranged up to 1 32 by the saline method and 1 128 by the enzyme method. The reactivity with enzyme (papain)-treated cells was stronger in all the cases except one. Serological specificity was established within the I i system in 16 cases. In 1 case the antibody was affected by the enzyme technique suggesting Pr specificity and in 1 case antibody specificity was not clear

Warm-Reacting Autoantibodies (5 Cases)

All these five sera reacted only at 37 C by the enzyme and by the indirect Coombs techniques. The titre ranged from 1 8 to 1 256 4 of these cases displayed their serological specificity within the Rh system and in the 5th case the specificity was not clear

Biphasic Autoantibodies (32 Cases)

The sera in this category were heterogeneous in their reactivity 30 out of 32 cases had saline reactivity at 6 C as well as at 37 C, which include 2 cases with strong saline reacting antibody (titre 1 16–1.32) at

Table III. Serological specificities involved in various types of autoantibodies

Auto-antibodies		I-i/Pr system							Rh system					Unclear specificity
		I^D	I	$\sim I^T$	HI	IP	i	~Pr	~D-like	~C lik		c	~pdl	
Auto-cold	18	3	7	3	1	1	1	1						1
Biphasic														
Predominantly cold	15	3	5	5			1							1
Predominantly warm	9								1	1	1	2	2	2
Rest	8								1					7
Auto-warm	5								1			2	1	1
Total	55	6	12	8	1	1	2	1	3	1	1	4	3	12

nol and all the six sera showed dual specificity of IgG+IgM. Interestingly 4 of these 6 cases had specificity within the Rh system. Among the rest of the biphasic autoantibodies (n = 8), 1 showed IgM and the remaining 7 had dual IgG + IgM specificity. All these sera were serologically nonspecific, except 1 which showed Rh specificity.

Correlation of immunoglobulin nature and serological specificity is shown in table II. The findings suggest that dual immunoglobulin specificity was more common in those biphasic antibodies which were not predominantly cold reacting. Interestingly all the predominantly cold-reacting biphasic antibodies had IgM specificity.

Levels of Immunoglobulins

There was a generalized increase in IgG and IgM immunoglobulin in more than 50% of the cases with autoimmune haemolytic anaemia. However IgA showed a slightly reduced level. The increase in IgG was more specific among the cases with auto-warm and biphasic showing predominantly warm antibodies. In more than half of these cases, the IgM level was elevated. Patients with auto-cold as well as biphasic predominantly cold antibodies showed a generalized increase of the IgM level. 10 out of 33 cases also showed an increased level of IgG. The titre of autoantibody had no correlation with the increased level of immunoglobulins.

Discussion

Autoantibodies of low titre are a common occurrence in normal individuals, but may also assume a pathological significance with *in vivo* coating of red cells causing haemolytic anaemia. Benign autoantibodies are mostly of the cold type and normally

Table II. Correlation of serological specificity and immunoglobulin nature of various types of autoimmune antibodies

Serological specificities	Types of autoantibodies	n	Type of immunoglobulin involved		
			IgG	IgM	IgG + M (dual)
I-I system	auto-cold	16	–	16	–
	biphasic[1]	14	–	14	–
Rh system	auto-warm	4	3	–	1
	biphasic[2]	8	1	–	7
Non-specific	auto-cold	1	–	1	–
	auto-warm	1	1	–	–
	biphasic[3]	10	1	1	8

[1] All predominantly cold type.
[2] 7 out of 8 cases belonged to the predominantly warm type.
[3] 1 predominantly cold, 2 predominantly warm and 7 unclassified biphasic type.

Rh null cells. The majority of the biphasic antibodies showing similar reactions at cold temperatures and at 37 C were nonspecific.

Nature of Immunoglobulins

All the 55 cases of autoantibodies were tested for immunoglobulin and 34 of them were tested for light chain specificities. Immunoglobulins were studied by (1) their sensitivity to 2 mercaptoethanol, and (2) the indirect Coombs test using monospecific anti IgG anti-IgM and anti IgA. Light chain specificity was ascertained by the indirect Coombs test using specific anti-kappa and anti-lambda antisera. Confirmation of the immunoglobulin characteristic was also made by passive haemagglutination inhibition of monospecific antiglobulin sera on eluate preparation of autoantibodies. The findings are given in table I All the 18 cold autoantibodies showed IgM specificity as confirmed by 2 mercaptoethanol as well as monospecific sera. The eluates also showed IgM specificity The light chain studies were carried out on 10 cases, wherein the majority had kappa specificity except for one which had dual specificity of both kappa and lambda. Among 5 cases of warm autoantibodies, 4 belonged to IgG and the 1 had dual specificity of IgG + IgM. This particular case had an antibody with Rh specificity Light chain studies were done in 2 cases, of which 1 case with no clear specificity gave reactions with both kappa and lambda. Among the biphasic antibodies the cases with the predominantly cold-reacting type were IgM type except for 1 who was partially affected by 2-mercaptoethanol treatment, having a dual immunoglobulin specificity of IgG + IgM. This particular case did not show clear-cut serological specificity Among the predominantly warm type, most of the cases (6 out of 9) were partially affected by 2 mercaptoetha-

lin specificities of IgG + IgM were more of ten of the biphasic nature with unclear serological specificity Evidently even these cases have either a kappa or lambda clone involved since none among the 8 cases with dual immunoglobulin specificity had dual light chain specificity The distribution of kappa and lambda in the present series is more or less similar to that reported by other workers [7 10]

The abnormal synthesis of immunoglobulin in autoimmune haemolytic anaemia is accelerated as is shown by an increase in the immunoglobulin levels of the IgG and IgM types. Apparently those with the cold type tend to have slightly elevated IgM and with the warm type, elevated IgG These observations are similar to those reported by other workers [4] and are attributed to the tissue breakdown [6]

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Dr H. M. Bhatia, Deputy Director and O/C,
Blood Group Reference Centre,
Seth G. S. Medical College, Parel,
Bombay 4000 12 (India)

have a very low titre rarely exceeding 1 64 [3] However the low titre cold autoantibody with a high complement affinity may also be of pathological significance [2] In a variety of conditions, autoimmune haemolytic anaemia occurring secondary to malignancies or viral diseases is not uncommon and may be induced by the administration of certain drugs such as aldomate [8] In the present series of 55 cases of autoimmune haemolytic anaemia, 33% were due to cold autoantibody 9% to warm autoantibodies and 58% being biphasic reacting at 4 and 37 C. The nature of cold autoantibodies suggests their specificity within the I i system, while those with warm autoantibody specificity lie within the Rh system. The present study also provides evidence that the biphasic antibody with wide thermal range (6–37 C) are heterogenous by nature. As shown in figure 1 they could be classified as predominantly cold or predominantly warm The serological nature as well as immunoglobulin specificity of these biphasic antibodies clearly indicates that those which are predominantly of the cold type more often resemble the auto-cold type of antibody whereas those with the predominantly warm type showed the characteristics of the auto-warm type. A small number of biphasic autoantibodies (n = 8) with more or less equal reactivity at both the temperatures were indeed difficult to classify Interestingly most of the cases with predominantly cold type biphasic antibodies showed specificities within the I-i system, whereas those with the predominantly warm type had specificities within the Rh. The rest of the biphasic antibodies did not show clear specificities. It is likely that such cases as well as others showing no serological specificity may indeed possess multiple specificities involving the I i as the cold antibodies and the Rh as the warm antibodies. Further studies by selective absorption and elution experiments in order to characterise the specificities involved are in progress.

The immunoglobulin nature of cold autoantibodies was mainly of IgM while that of warm autoantibodies was IgG Those with biphasic antibodies and predominantly cold type again showed an IgM nature. However many of the cases of remaining biphasic autoantibodies had dual immunoglobulin specificities of IgG + IgM. This again confirms the nature of different antibodies with different immunoglobulin specificities.

It is likely that the autoimmune phenomenon of pathological significance has a sequential order of autoimmune changes from auto-cold (IgM), to biphasic cold type (IgM) to biphasic unclassified (IgM + G), to biphasic warm type (IgG + M) and to auto-warm (IgG) It is also likely that various other primary conditions like malignancy may substantiate further to the development of autoimmune haemolytic anaemia. This is supported from the findings of 7 out of 32 cases of biphasic autoantibodies which had malignancies as the primary cause However the majority of these malignant conditions with biphasic autoantibodies are predominantly cold type and in all of them complement mediation played a significant role.

The nature of autoantibodies of the cold, biphasic, and warm types is also reflected by the immunoglobulin type It is apparent from the findings of the immunoglobulin light chain studies that the single abnormal clone of one particular immunoglobulin type with either kappa or lambda is involved. Those with the dual immunoglobu-

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AS-BI phosphatase substrate (Sigma) + fast red violet LB (Sigma). For each case 300 PMNs were visually graded from 0 to 3 by scoring method according to intensity of the reaction as follows: 0 = no granules; 1 = few scattered granules; 2 = many regularly dispersed granules; 3 = numerous granules covering the whole cytoplasm surface. (c) A hemacytometric study was performed on the red and white blood cells with

Table I. Hemacytometric data for parents of trisomy 21 children (a n = 106) and of normal children (b n = 220)

Parameter		Mean a/b	Standard deviation a/b	Significance
RBC 10^6/mm³	M	4.57/4.73	0.35/0.37	NS
	F	5.12/5.03	0.37/0.35	NS
Hgb, g/100 ml	M	13.71/14.10	1.10/1.0	NS
	F	15.80/15.44	1.0 /1.20	NS
WBC, 10^3/mm³	M	6.20/6.76	1.56/1.85	NS
	F	6.83/6.55	2.01/1.71	NS
PMNs, 10^3/mm³	M	3.94/4.46	1.45/1.60	NS
	F	4.29/3.97	1.84/1.25	NS
Lymphocytes, 10^3/mm³	M	1.72/1.83	0.52/0.67	NS
	F	1.92/1.95	0.55/0.70	NS
Monocytes, 10^3/mm³	M	0.36/0.35	0.11/0.19	NS
	F	0.48/0.48	0.24/0.23	NS

M = Mothers F = fathers NS = not significant.

Coulter Counter S and a differential WHO count was done for 200 cells on smears stained with May-Grünwald-Giemsa.

The data thus obtained were studied statistically for range, mean, variance and standard deviation.

Results

Karyotype analysis showed normal results in 322 cases. 4 parents of trisomy 21 children showed the following modifications: 46,XX/47,XXX (6%) 46,XX/46,XX,ctb(1)(p22) (30%) 46,XY,inv(6)(p25q13) 46,XY 1qh+

The karyotypes of the 53 children with a free, homogeneous trisomy 21 were established during an earlier study [6]

The hemacytometric study showed nonsignificant differences in the number of white cells, red cells, and hemoglobin between the parents of trisomy 21 children and the control population. However certain numerical modifications seem interesting: (a) the decrease in the number/mm³ of PMNs and lymphocytes in the mothers, and (b) the increase in the number/mm³ of PMNs in the fathers (table I)

The NAP are increased significantly in the parents of trisomy 21 children ($p < 0.05$ table II)

Table II. Neutrophil alkaline phosphatase activity in mothers and fathers of trisomy 21 children and in parents of normal children

	Parents of trisomy 21 children	Parents of normal children	p value
Mothers	52.51 ± 20.35 (= 53)	38.81 ± 21.60 (n = 110)	< 0.05*
Fathers	43.20 ± 26.09 (n = 53)	30.53 ± 20.42 (n = 110)	< 0.05

$0.03 < p < 0.05$.

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Increase of Neutrophil Alkaline Phosphatase in the Parents of Trisomy 21 Children

Hematological and Cytogenetic Studies

J. Grozdea, G. Bourrouillou, C. Mounie and P. Colombies

Inserm, U 100 and Laboratoire de Cytogénétique, CHU Purpan, Toulouse

Key Words. Hemacytometry · Karyotype · Neutrophil alkaline phosphatase · Parents of trisomy 21 children

Abstract. A comparative study of karyotypes, hematological variables and neutrophil alkaline phosphatase (NAP) was performed in 106 parents (53 couples) of children with free, homogeneous trisomy 21 and of 220 parents (110 couples) of normal children. In the parents of trisomy 21 children we found a significant increase of the NAP ($p < 0.05$) and a nonsignificant difference in the number of white blood cells. In 4 cases, we also found an anomaly in the karyotype. A modification of the metabolism of the neutrophils seems possible.

Introduction

It is well known that the NAP is increased in trisomy 21 children [1]. However the genetic localization and the mechanism which induces these enzymatic anomalies are as yet unknown.

NAP activity was previously found increased in women over 40 years of age. In this age group there is a high incidence of trisomy 21 children born [4].

These statistical data as well as our earlier observations [6] caused us to study the NAP, karyotype, and hemacytometry in the parents of trisomy 21 children.

Subjects and Methods

106 parents of trisomy 21 children (53 couples) aged 17–48 years (mean age 29 years) and 220 controls (110 couples) aged 19–42 years (mean age 28.5 years), parents of to 5 children, all apparently normal and healthy, were studied.

The tests performed were: (a) A karyotype study after culture of peripheral blood [5] and enzymatic denaturation according to the technique of *Seabright* [11]. For each case, 45–50 cells were analysed. (b) A semiquantitative study of the alkaline phosphatases in the polymorphonuclear neutrophils (PMNs) following the technique of *Kaplow* [9]. Smears of peripheral blood were fixed in a solution of methanolformaldehyde, washed, dried, and treated for 10 min with a β-naphthol

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J. Grozdea, Inserm, U-100, CHU Purpan, F-31052 Toulouse Cédex (France)

Discussion

In this study the parents of trisomy 21 children present a karyotype of 46 chromosomes without anomaly except for 4 cases. The chromosomal modifications in the population under study are comparable to data found in the literature [2, 3]

The parents of trisomy 21 offspring seem to carry several minor hematological anomalies The number of circulating granulocytes was found increased in the father decreased in the mother The mothers also showed a decrease in the number of lymphocytes. These results seem to us interesting because in an earlier study we found a significant decrease in the number of circulating lymphocytes in newborn trisomy 21 infants [7] Another modification in the granulocytes of parents of trisomy 21 children is the significant increase of NAP ($p < 0.05$) This enzyme level can be related to the even higher level found in trisomy 21 children.

NAP activity was previously found increased in various physiological and pathological states active granulopoiesis with circulating young PMNs [10] bacterial infection [8] increased IgG [12] pregnancy [7] and state of stress [13]

The high NAP levels we found in the parents of trisomy 21 children cannot be included in these categories In these parents, there seems to be a modification of the metabolism of PMNs Further study of the PMNs of these subjects is now being carried out by functional tests and culture techniques.

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J Gruzden, Inserm, U-100, CHU Purpan, F-31052 Toulouse Cédex (France)

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Beta°-Thalassemia/Hb E Association

Hemoglobin Synthesis in Blood Reticulocytes and Bone Marrow Cells Fractionated by Density Gradient and in Blood Erythroid Colonies in Culture

U Testa A Dubart N Hinard F Galacteros W Vainchenker P Rouyer Fessard Y Beuzard and J Rosa

Unité de Recherches sur les Anémies, Inserm U-91 Hôpital Henri Mondor Créteil

Key Words. β-Thalassemia Erythroid cell culture Globin chain synthesis Hb E

Abstract. Hemoglobin synthesis in a 34-year-old man of Laotian ancestry with Hb E/β° thalassemia was studied. Hemoglobin electrophoresis exhibited only Hb F and Hb E. Reticulocytes and bone marrow cells showed no Hb A synthesis. The proportion of Hb F increased (from 14 to 50%) with the density of RBCs fractionated on a discontinuous gradient of Stractan. Reticulocytes were also heterogeneous with regard to their density and their Hb F synthesis. the proportion of Hb F synthesis increased with their density Fractionation of bone marrow cells by density on a discontinuous gradient of Stractan provided populations of cells at different stages of maturation, the more mature having the greater density Study of hemoglobin synthesis in bone marrow cells indicated that the proportion of γ-chains synthesized increased and the pool of free α-chains decreased with erythroblast maturation In contrast, the proportion of βE-chains compared to α-chain synthesis remained constantly low These results provide evidence that the increased amount of Hb F found in the blood of the patient was mostly due to the positive selection, in bone marrow and in peripheral blood of cells containing Hb F In contrast, colonies of early erythroid precursors derived from the blood of the patient exhibited an enormous reactivation of Hb F synthesis in culture. The low proportion of βE-chain synthesis (about 30% of α-chain synthesis) remained unchanged during the erythroid maturation of most immature erythroblasts to reticulocytes. The combination of two thalassemic alleles, one totally deficient (β°) the other partially deficient (βE) can explain the clinical severity of the association β° thalassemia/Hb E.

Introduction

The β° thalassemia/Hb E association (β° thal/Hb E) represents a combination of the two most frequent hemoglobinopathies of Southeast Asia. This syndrome is characterized by a clinical picture similar to Cooley's anemia, although milder forms have been described [8] As in other severe thalassemic syndromes, the proportion of Hb F

found in the peripheral blood is increased and is heterogeneously distributed among RBCs. This phenomenon was tentatively attributed to the preferential survival of the cells containing Hb F during erythroid maturation [31]. However until now no direct evidence has been found to support this hypothesis. It is known that only thalassemic RBCs containing Hb F are able to survive longer than the cells which do not contain Hb F [13].

In the present study hemoglobin synthesis was studied in erythroblasts, reticulocytes and RBCs of a patient with β^0-thal/Hb E association. A progressive increase in Hb F synthesis was observed during the erythroid maturation from proerythroblast to reticulocyte, affording direct and clear evidence in favor of the above-mentioned hypothesis. In contrast, in culture of early erythroid precursors (BFU-Es) a true stimulation of Hb F synthesis was observed.

Materials and Methods

Hematological Studies

All the usual hematological parameters were determined by standard techniques. The ratio of Hb E and Hb F in peripheral blood was evaluated by electrophoresis on cellulose acetate or by isoelectric focusing [1]. The distribution of Hb F in single erythrocytes was estimated by the acid elution method [16].

Separation of Erythrocytes and Bone Marrow Cells by Density Gradients

RBCs were separated by ultracentrifugation on discontinuous gradient of Stractan II, purified and prepared as described by *Corash et al.* [7].

For RBC fractionation, 8 different fractions of Stractan (density from 1.065 to 1.110) were layered in 12-ml nitrocellulose tube (Beckman, SW 41); 0.2 ml of cells, previously incubated with ³H leucine as described below were suspended in 1 ml of BSKG buffer (phosphate buffer 10 mM pH 7.40, glucose 0.2%, KCl 5 mM and NaCl in an amount required to obtain 291 mOsm) and layered on the gradient. The cells were centrifuged at 35 000 g in a Beckman rotor SW 41 in an L 50 Beckman ultracentrifuge for 50 min at 4 °C. At the end of centrifugation, the cells were carefully removed by aspiration with Pasteur pipettes and then extensively washed in cold saline solution. One part of the cells was lysed and submitted to electrophoresis for use in hemoglobin analysis. The proportion of reticulocytes in the different fractions was determined by the new methylene blue staining and by the presence of radioactivity incorporated in the hemoglobin fractions of samples of blood incubated with ³H-leucine prior to the fractionation. For bone marrow cells, a different gradient was used (density from 1.045 to 1.090). After centrifugation, the cells were extensively washed in NCTC 109 medium. One aliquot was frozen at −80 °C until use; another aliquot was smeared and stained to determine the proportions of cells of different types and different maturation stages in each fraction.

Globin Chain Synthesis in Reticulocytes

Blood was drawn on heparin, washed 4 times with sterile NCTC 109 medium made leucine free (Eurobio, Paris, France) and then incubated in the same medium containing 200 µCi of ³H-leucine (CEA, Saclay, France) at 37 °C for 2 h. The globins were prepared by acid acetone precipitation. The radioactivity incorporated into the globin chains was estimated by chromatography on carboxymethyl cellulose in urea using the method of *Clegg et al.* [6] slightly modified by *Testa et al.* [25].

Globin Chain Synthesis in Bone Marrow

Bone marrow cells were dissociated by gentle aspiration through needle, washed 4 times and incubated with ³H-leucine as described above.

The cells were lysed by distilled water and the hemoglobin was purified from nonheme proteins by affinity chromatography on Sepharose-haptoglobin [26]. Before the binding to hapto-Sepharose, the lysate was preincubated with βSH-chains purified according to *Bucci and Fronticelli* [4] and *Yip et al.* [27] in order to convert all the α-free chains present in the sample into hemoglobin. The globin chains were then separated as described for the reticulocytes.

Evaluation of the Pool of Free α-Hemoglobin Chains

For the evaluation of the pool of free α-chains, the cells were incubated as previously described and the incubation was stopped at 20 60 or 120 min. The radioactivity present in the pool of free α-hemoglobin chains was estimated by electrophoresis on cellulose acetate (Cellogel, Chemetron, Milan, Italy) as previously described [2]. In addition, this method also allowed the separation and measurement of the radioactivity incorporated in Hb E and Hb F

Evaluation of the α-Chain Pool in Bone Marrow

Bone marrow cells were incubated as previously described and aliquots were removed at 20, 60 and 120 min.

Three different aliquots of the membrane-free hemolysate (about 100 μg of hemoglobin) of each sample were submitted to electrophoresis on cellulose acetate: the first was not modified, the second was preincubated for 60 min at 4 °C with β-SH chains to convert all the free α-chains into Hb the third was first incubated for 60 min at 4 °C with βSH-chains and then for the same period time and at the same temperature with purified human haptoglobin. At the end of the migration, the strip of cellulose acetate was stained with amido black and was then transversally sliced at 0.5-cm intervals and processed as previously described [2] for the evaluation of the radioactivity in each fraction. The comparison of the radioactivity in corresponding fractions of the first and of the second samples made it possible to estimate the proportion of radioactivity contained in the pool of free α-chains comparison between the first and the third samples allowed correct evaluation of the radioactivity in the hemoglobin fractions. One example is given in figure 1

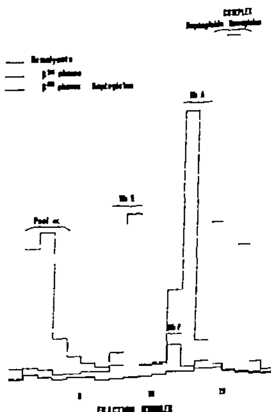


Fig. 1 Radioactivity pattern obtained upon electrophoresis of the hemoglobin of the proband's bone marrow cells on cellulose acetate strip

Cultures

Mononuclear cells from the blood of the patient were isolated by centrifugation on a cushion of Ficoll-metrizoate (Lymphoprep Nyegaard, Oslo) The cells were washed three times in α-medium (Eurobio, Paris, France) and the plasma clot cultures established in 35-mm Falcon Petri dishes containing 1×10^6 nucleated cells in 1 ml [19]. Slight modifications of the technique were used, as previously described [28] α-medium replaced the NCTC 109 medium, human AB serum was used instead of fetal calf serum and $CaCl_2$ was added at a final concentration of 0.026 mg/ml. Bovine embryo extract was omitted. Erythropoietin step III preparation (Connaught Research Laboratories, Toronto, Canada) was added at zero time at a concentration of 3 U/ml. The cultures were then incubated for 11–16 days in 6 different Petri dishes.

On the 7th or 14th day the cultures were stained directly in the Petri dishes. The erythroblastic bursts were identified by staining of the pseudoperoxidase activity of hemoglobin [18]. Quantitation was done under an inverted microscope at 40× magnification.

At days 11, 13 and 15 100 μCi of H leucine (50 Ci/mM Commissariat à l'Energie Atomique, Saclay France) was added in 0.3 ml of NCTC 109 medium (leucine free; Eurobio) to one culture dish which had been incubated to produced erythroid

colonies. Incubation was terminated 24 h later by digestion of the clot with pronase (1 mg/ml Calbiochem, San Diego, Calif.) for 10 min at room temperature: such treatment allowed the recovery of all the cells from one dish. Red blood cells were added as carrier when necessary. The cells were extensively washed with Hanks' medium and then with sodium chloride (0.15 M), and then frozen at –80 °C until use.

After purification of hemoglobin by affinity chromatography on Sepharose-haptoglobin as previously reported [26, 29], the proportion of radioactivity incorporated into the globin chains was evaluated by chromatography on carboxymethyl cellulose in urea.

Results

Case Report

The patient of Laotian ancestry showed a clinical syndrome of β-thalassemia intermedia. He remained transfusion-free until the age of 33 years. At this time he underwent clinical observation for a mediastinal opacity which, at anatopathological examination, was shown to be due to extramedullary erythropoiesis. A splenectomy was subsequently performed. The patient's hematological parameters are reported in table I.

Table I

	Propositus	Normal
Hb, g%	8.5	15.3 ± 1
PVC, %	27	46 ± 3
RBC, $10^6/\mu l$	4.65	5.4 ± 0.5
MCV, μm^3	60	85 ± 8
MCH, pg	18.5	28 ± 2.5
MCHC, %	32	33 ± 2.5
Reticulocytes, %	5–10	< 2
Serum iron, μg%	80–182	110 ± 35
Serum ferritin, μg/100 ml	150	12 ± 10
Serum haptoglobin, g/l	< 0.2	0.4–2
Hb F, %	36	< 1

Hemoglobin Synthesis in Peripheral Blood and Bone Marrow

Hemoglobin synthesis in peripheral blood showed an absence of βA-chain synthesis in reticulocytes (fig. 1); furthermore, the proportion of βE-chain with respect to α-chain was reduced (0.34) with respect to that expected for a β-chain in heterozygotes (0.5). Hemoglobin synthesis in bone marrow (fig. 2) was slightly more balanced, and the γ/α ratio was lower (0.09) than in reticulocytes (0.14). The results of the study of Hb synthesis are summarized in table II.

Proportion and Synthesis of Hemoglobin in RBCs Separated by Density

The proportion of Hb E and Hb F in different fractions of red blood cells was evaluated by electrophoresis and is reported in

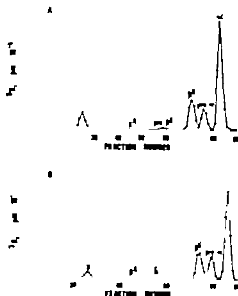


Fig. 2. Radioactivity profiles of globin chains from the proband' blood reticulocytes (A) and bone marrow cells (B).

Evaluation of the Pool of Free α-Hemoglobin Chains

For the evaluation of the pool of free α-chains, the cells were incubated as previously described and the incubation was stopped at 20 60 or 120 min The radioactivity present in the pool of free α hemoglobin chains was estimated by elec trophoresis on cellulose acetate (Cellogel, Chemetron, Milan, Italy) as previously described [2] In addition, this method also allowed the separation and measurement of the radioactivity incorporated in Hb E and Hb F

Evaluation of the α-Chain Pool in Bone Marrow

Bone marrow cells were incubated as previously described and aliquots were removed at 20, 60 and 120 min.

Three different aliquots of the membrane free hemolysate (about 100 μg of hemoglobin) of each sample were submitted to electrophoresis on cellulose acetate: the first was not modified, the second was preincubated for 60 min at 4 °C with β-SH chains to convert all the free α-chains into Hb, the third was first incubated for 60 min at 4 C with βSH-chains and then for the same period time and at the same temperature with purified human haptoglobin. At the end of the migration, the strip of cellulose acetate was stained with amido black and was then transversally sliced at 0.5-cm intervals and processed as previously described [2] for the evaluation of the radioactivity in each fraction. The comparison of the radioactivity in corre sponding fractions of the first and of the second samples made it possible to estimate the propor tion of radioactivity contained in the pool of free α-chains, comparison between the first and the third samples allowed correct evaluation of the radioactivity in the hemoglobin fractions. One ex ample is gvien in figure 1.

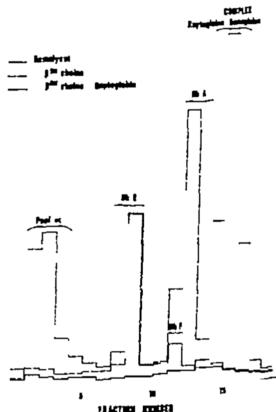


Fig. 1. Radioactivity pattern obtained upon electrophoresis of the hemoglobin of the proband's bone marrow cells on cellulose acetate strip.

Cultures

Mononuclear cells from the blood of the patient were isolated by centrifugation on a cushion of Ficoll-metrizoate (Lymphoprep Nyegaard, Oslo) The cells were washed three times in α-medium (Eurobio, Paris, France) and the plasma clot cultures established in 35-mm Falcon Petri dishes containing 1×10^{6} nucleated cells in 1 ml [19] Slight modifications of the technique were used, as previously described [28] α-medium replaced the NCTC 109 medium, human AB serum was used instead of fetal calf serum and $CaCl_2$ was added at a final concentration of 0.026 mg/ml. Bovine embryo extract was omitted. Erythropoietin step III preparation (Connaught Research Laboratories, Toronto, Canada) was added at zero time at a concentration of 3 U/ml. The cultures were then incubated for 11–16 days in 6 different Petri dishes.

On the 7th or 14th day the cultures were stained directly in the Petri dishes. The erythroblastic bursts were identified by staining of the pseudoperoxidase activity of hemoglobin [18]. Quantitation was done under an inverted microscope at 40× magnification.

At days 11 13 and 15, 100 μCi of ^{3}H leucine (50 Ci/mM Commissariat à l'Energie Atomique, Saclay France) was added in 0.3 ml of NCTC 109 medium (leucine-free Eurobio) to one culture dish which had been incubated to produced erythroid

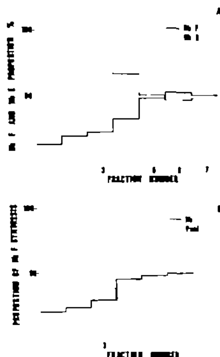


Fig. 3. Proportion of Hb E and Hb F determined in different fractions of RBCs separated by density on discontinuous gradient of Stractan (A). Proportion of radioactivity into Hb F and in the pool of free α-chains in different fractions of RBCs separated by density on discontinuous gradient of Stractan (B).

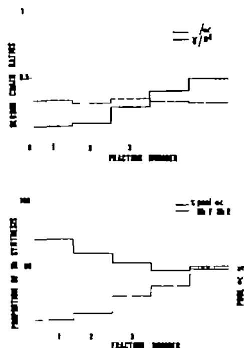


Fig. 4. Proportion of radioactivity in Hb E, Hb F and in the pool of free α-chains in different fractions of bone marrow cells separated by density (lower panel). Globin chain ratios (βE/α and γ/βE) in different fractions of bone marrow cells separated by density. Fraction 1 was composed almost exclusively of proerythroblasts and basophilic erythroblasts, fraction 2 by basophilic erythroblasts, fraction 3 by polychromatophilic and orthochromatic erythroblasts, fraction 4 by orthochromatic erythroblasts, and fraction 5 by reticulocytes.

Table V. Globin chain synthesis in bone marrow cells subsequently separated by density

Fraction	βE/α	γ/α	γ/βE	Non-α
1	0.325	0.035	0.13	0.355
2	0.3	0.04	0.14	0.344
3	0.33	0.09	0.27	0.42
4	0.31	0.12	0.39	0.43
Blood reticulocytes	0.30	0.14	0.48	0.44

Table VI. Hemoglobin synthesis and pool of free α-chains in bone marrow cells separated by density

Fraction	Hb F/Hb E	% pool
1	0.09	71
2	0.14	60
3	0.27	53
4	0.35	46
Reticulocytes	0.5	48

Table II. Hemoglobin synthesis in peripheral blood and bone marrow

a Globin chain *chromatography on CM-cellulose*

	βE/α	γ/α	δ/α	Non-α
Peripheral blood	0.30	0.14	0	0.44
Bone marrow	0.34	0.09	0.03	0.46

b Electrophoresis on cellulose acetate

		Hb E/Hb tot.	Hb F/Hb tot.	Hb F/Hb F	% pool
Peripheral blood	20 min	0.66	0.34	0.50	57
	120 min	0.67	0.33	0.50	43
Bone marrow	20 min	0.77	0.23	0.30	23
	120 min	0.80	0.20	0.25	49

Table III. Proportions of Hb F and of Hb E in fractions of peripheral red blood cells fractionated by density gradient

Fraction	% Hb E	% Hb F
1	86	14
2	80	20
3	78	22
4	68	32
5	49	51
6	48	52
7	50	50
Unfractionated RBCs	63	37

Table IV. Radioactivity of the pool of free α-hemoglobin chains and proportions of Hb E and Hb F synthesized in reticulocytes of different density

Fraction	% Hb E	% Hb F	% pool
1	79	21	67
2	75	25	42
3	71	29	38
4	55	45	30
5	51	49	24
6	50	50	22
Whole blood	67	33	41

table III. A progressive increase in Hb F proportion from low to high-density red blood cells was obtained. The hemoglobin synthesis in the reticulocytes present in the same fractions showed the same phenomenon associated with a concomitant reduction of the pool of free α-chains (table IV). These results are illustrated in figure 3

Hemoglobin Synthesis in Bone Marrow Cells Separated by Density

Bone marrow cells were separated according to their density by centrifugation on a discontinuous gradient of Stractan. Each fraction contained populations of erythroblasts almost homogeneous for their maturation, making it possible to study hemoglobin synthesis according to the maturation of erythroid cells. The study of globin chain synthesis is summarized in table V and figure 4. The results showed an increasing β/α ratio with a progressively more balanced hemoglobin synthesis during maturation from proerythroblasts to acidophil erythroblasts. In contrast, a constant βE/α ratio was observed

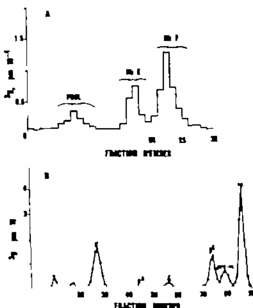


Fig. 6. A Radioactivity pattern obtained upon electrophoresis of the hemoglobin of the proband' BFU-E colonies on cellulose acetate strip B Radioactivity profiles of globin chains from the proband' BFU-E separated by chromatography on carboxymethyl cellulose in urea

Table VII. Hemoglobin synthesis in freshly drawn cells from bone marrow and [ ] blood BFU-E colonies at different days of culture

	Non-α/	βE/α	δ	γ/α
Fresh bone marrow cells	0.46	0.29	0.03	0.14
BFU-E colonies (11 days)	0.81	0.35	0.05	0.41
BFU-E colonies (13 days)	0.78	0.32	0.03	0.43
BFU-E colonies (15 days)	0.80	0.33	0.02	0.45

normal than that in the peripheral blood [3 12] Other workers, however have not demonstrated such a striking difference between the globin synthesis in marrow and that in the peripheral blood of patients homozygous for β-thalassemia [14 20, 24] The only available information on hemoglobin synthesis during erythroid maturation is from the work of *Kim et al* [15] and *Wood et al.* [33] 4 subjects homozygous for β-thalassemia were studied by the former group, and a progressive increase in the non-α/ ratio was observed during erythroid maturation In contrast, no consistent changes in the proportion of γ-globin synthesis was observed. The latter group, studying a β-thalassemia homozygote, reported a decrease in the β/α ratio and an increase in the γ/non-α-chain synthesis ratio with erythroid maturation.

In the present work, we studied a patient heterozygous for β -thalassemia and Hb E who exhibited a significant increase in the proportion of Hb F in peripheral blood. The synthesis of the βE-chain remained constant during the different steps of erythroid maturation from basophilic erythroblast to blood reticulocyte. Consequently it can be used as a control to which all the other parameters studied can be referred. The Hb F in erythrocytes was heterogeneously distributed, as evaluated by Kleihauer's method this also is the case in homozygous β⁺-thalassemia. In order to evaluate the proportion of Hb F contained in red blood cells of different ages, we separated them according to density Density increased with aging, the old cells contained more Hb F than young cells. This result was in full agreement with the findings reported by *Loukopoulos and Fessas* [17] who showed that F erythrocytes in thalassemia have a higher density and probably a preferential survival [11 13]. In addition, we obtained evidence for the het

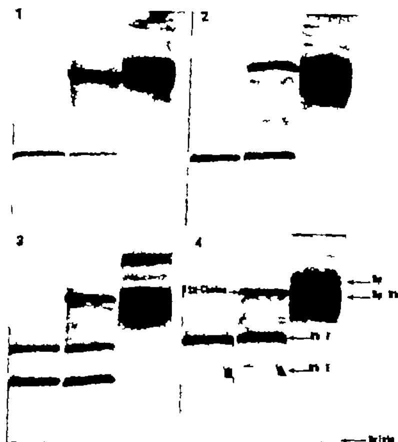


Fig. 5. Electrophoretic pattern on acetate cellulose (pH 8.7) given by the hemolysate from proband's bone marrow cells fractionated by density amido black staining. Fractions 1–4 as in figure 4. Electrophoretic patterns of hemolysate (left), after addition of purified β-chains (middle), and after addition of purified β-chains and human haptoglobin (right). Hp = Haptoglobin. Hp-Hb = haptoglobin-hemoglobin complex.

The results obtained by electrophoresis of hemolysates indicated a progressive increase in the Hb F/Hb E ratio and a decrease in the pool of free α-chains (table VI fig 4 5) which correspond to different stages of terminal erythroid differentiation (table VII)

Hemoglobin Synthesis in Erythroid Colonies

The study of hemoglobin synthesis in culture revealed a more balanced globin chain synthesis than in freshly drawn cells (fig. 6) This was due to an important increase in the porportion of γ-chain synthesis in culture since the γ/α ratio increased from 0 09 in fresh bone marrow cells to 0 41 in erythroid culture in contrast, the $\beta E/\alpha$ ratio remained unchanged

The γ/α and $\beta E/\alpha$ ratios remained constant at three different days of culture

Discussion

While the hemoglobin synthesis during erytroid maturation in people with the β-thalassemia trait is well documented [5 23 32] few studies have been performed in cases of homozygous β-thalassemia. In homozygous β-thalassemia, there is either a total absence of βA-chain radioactivity or a marked deficiency in βA-chain radioactivity with a β/α ratio of 0 1–0.3 [30] as measured in reticulocytes. When globin synthesis was studied in marrow of patients homozygous for β-thalassemia, a still abnormal β/α synthetic ratio was observed, but it was closer to

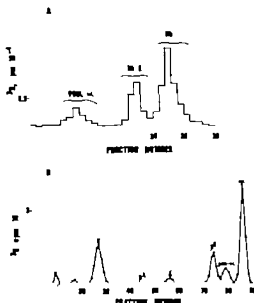


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Table VII. Hemoglobin synthesis in freshly drawn cells from bone marrow and in blood BFU-E colonies at different days of culture

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In the present work, we studied a patient heterozygous for β^0-thalassemia and Hb E who exhibited a significant increase in the proportion of Hb F in peripheral blood. The synthesis of the βE-chain remained constant during the different steps of erythroid maturation from basophilic erythroblast to blood reticulocyte Consequently it can be used as a control to which all the other par ameters studied can be referred The Hb F in erythrocytes was heterogeneously distributed, as evaluated by Kleihauer s method this also is the case in homozygous β^+-thalassemia. In order to evaluate the proportion of Hb F contained in red blood cells of dif ferent ages, we separated them according to density Density increased with aging, the old cells contained more Hb F than young cells. This result was in full agreement w th the findings reported by *Loukopoulos and Fessas* [17] who showed that F erythrocytes in thalassemia have a higher density and probably a preferential survival [11 13] In addition, we obtained evidence for the het

erogeneity of reticulocytes density by the same gradient technique This heterogeneity was constantly observed in thalassemic subjects and never for normal individuals who exhibited reticulocytes with homogeneous density [25] For the propositus, the analysis of hemoglobin synthesis in reticulocytes of different densities showed that the proportion of Hb F synthesis was increased in the most dense reticulocytes. In addition where Hb F synthesis was higher a smaller pool of free α-chains was observed. Similar results were obtained with reticulocytes from patients homozygous for β-thalassemia [25]

It can be hypothesized that a continual process of cell selection occurs during erythroid maturation in the bone marrow of thalassemic subjects with only those cells with the least chain imbalance (more fetal hemoglobin) surviving to enter the peripheral blood [31] To test this hypothesis we studied hemoglobin synthesis in erythroid cells fractionated according to their maturation. the γ/α ratio increased from 0 03 in proerythroblasts to 0 14 in blood reticulocytes A concomitant reduction of globin chain imbalance was found, as shown by the non-α/α ratio of globin chain synthesis and by the evaluation of the pool of free α-chains in contrast the $\beta E/\alpha$ ratio remained constant. Furthermore in the fraction composed mostly of proerythroblasts and basophilic erythroblasts, the γ/α ratio was 0 04 and may represent the true γ-chain synthesis in erythroid cells not yet selected by the ineffective erythropoiesis. This value for the γ/α ratio is higher than that observed in the blood of normal individuals and could be explained if we take into account that a stimulated erythropoiesis is able to increase Hb F production [9] It can be concluded that the increased proportion of Hb F in the present subject occurs through two different mechanisms first, a positive selection of erythroid cells containing Hb F second and to a lesser extent, the stimulation of γ-gene expression induced by the stimulated erythropoiesis. It is probable that the two mechanisms act similarly in other severe β-thalassemia syndromes and that γ-chain stimulation is variable in its expression from one subject to another

In contrast, in culture of early erythroid precursors (BFU E) from the blood of the patient, a great reactivation of γ-chain synthesis (γ/α ratio = 0 41) was observed. This increase of Hb F synthesis in culture is not the consequence of a positive selection of cells synthesizing Hb F because similar γ/α ratios were found at days 11 and 15 of culture which for most of the colonies corresponds to the period of time necessary for the maturation from immature erythroblasts to reticulocytes In addition, the $\beta E/\alpha$ ratio remained constant during this period and was identical to that observed in fresh bone marrow cells or in blood reticulocytes. This result clearly shows that the increased synthesis of γ-chains in culture is made on the top of βE-chain synthesis. It is very important to understand why this enormous potential for γ-chain synthesis is not expressed *in vivo* when it could be advantageous for the patient. It is probable that this discrepancy is dependent on the different pattern of erythroid differentiation *in vivo* and in culture since the reactivation of γ-gene expression in culture appears to be linked to the process of erythroid differentiation [22]

In addition we found a constant and reduced synthesis of the βE allele (corresponding to only 60% of the normal βA allele) during the different steps of erythroid

maturation. The similar level of βE synthesis in immature erythroblasts and in reticulocytes suggests that the βE mRNA is not unstable as found for the δ or Lepore globin mRNA. The results, like those of others in the literature [10 21] indicate clearly that Hb E is not an unstable hemoglobin but probably a hemoglobin synthesized at a reduced rate it could be tentatively classified as a moderate thalassemic allele. The combination of two thalassemic alleles, one totally deficient (β^0) and the other partially deficient (βE), could explain the clinical severity of the association β^0-thal/Hb E.

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Dr U Testa,
Unité de Recherches sur les Anémies,
INSERM U-91 Hôpital Henri-Mondor
F-94010 Créteil (France)

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Ultrastructural Studies of an Unusual Variant of Congenital Dyserythropoietic Anaemia Type II

Catharine L. Dewar and Ray M. Lowenthal

Department of Medicine, University of Tasmania Clinical School, Hobart, Tasmania

Key Words. CDA II Electron microscopy Erythrocytes Granulocytes Kell blood group Platelets

Abstract. Electron microscopy of a new variant of congenital dyserythropoietic anaemia type II has yielded several unexpected results. The remarkable features include the relatively high percentage of erythrocytes with peripheral cisternae, the involvement of all bone marrow precursor cells, and the presence of ultrastructural defects in the granulocytes and platelets and on the cell surface of the erythrocytes. Experiments with vinblastine showed *that this drug was capable of producing (in vitro)* strikingly similar ultrastructural defects in healthy erythrocytes.

The ultrastructural features of typical cases of congenital dyserythropoietic anaemia (CDA) type II are highlighted by the presence of peripheral cisternae ('double membranes') underneath the erythrocyte cell surface [5–7]. In the present study we describe the distinctive ultrastructural features of the blood and bone marrow cells of a patient with a new variant of CDA II. The full clinical investigation of this extraordinary patient has been published elsewhere [14]. In brief, the patient is the product of a first-cousin marriage, and his main clinical problems are anaemia, severe tophaceous gout and probable haemosiderosis with cardiomyopathy. Representative blood values include Hb 9.5 g/dl, MCV 114 fl, reticulocytes 6%, and nucleated red blood cells 5.5×10^9/L. He has a very low serum cholesterol level and his erythrocytes, in addition to their abnormal appearance, are of the rare Kell genotype Kp^a/Kp^a. Despite the typical II status, the patient's erythrocytes are missing the Hempas antigen characteristic of CDA II [14].

Material and Methods

Scanning electron microscopy was performed as described previously [4]. For transmission electron microscopy whole-blood (0.1 ml) anticoagulated with heparin, bone marrow (sternal aspiration) or isolated granulocytes [3] were fixed in glutaraldehyde (2.5 g/dl, buffered to pH 7.2 with sodium cacodylate, 2.1 g/dl) for 4 h at 25 °C. The fixed cells were then washed in sucrose/cacodylate buffer [17] and post-fixed in OsO (1 g/dl in 2.1 g/dl sodium cacodylate) for 30 min. The cells were washed again, then dehydrated in graded series of ethanols and embedded according to *Spurr*

[18] Sections were cut with an LKB III ultramicrotome stained with uranyl acetate [15] and lead citrate [16], and examined in a Hitachi HS-7S electron microscope. The ultrastructure of the erythrocytes from the patient and a healthy control was also examined after incubation of the erythrocytes (in two separate experiments) with 10 μmol/l vinblastine (VBL) for 30 min at 37 C.

Results

Scanning electron microscopy showed a bizarre erythrocyte morphology in the majority of cells examined. Occasionally cells with pits or depressions in the membrane were also seen (fig. 1a).

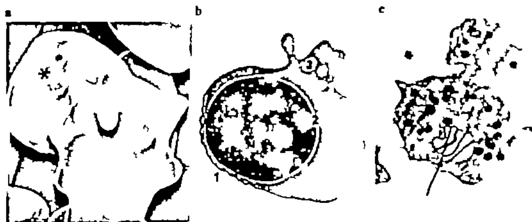


Fig. 1. Scanning and transmission electron microscopy a Bizarre erythrocyte morphology including membrane pits and depressions (*) × 4,500 b Peripheral blood erythroblast showing peripheral cisterna (1), widened nuclear pores (2), and cytoplasmic vacuoles (3). × 5,000. c Platelet showing vacuoles and stacks of cytoplasmic cisternae (arrow) × 9,600



Fig. 2. Transmission electron microscopy a Fingerlike projection containing the cisternae (arrow) × 16,400 b Granulocytes showing peripheral cisternae (1) peri nuclear cisternae (2), widened nuclear pores (3) and a phagocytic vacuole (4). × 5,500.

Transmission electron microscopy of the peripheral blood revealed that 5.7% of the 1,036 erythrocytes examined contained peripheral cisternae. All nucleated RBC in circulation also contained the cisternae. Fingerlike projections from the erythrocytes often contained the cisternae (fig. 2a). Layers of cisternae, internal cisternae and cisternae circumscribing entire cells could be found. Other cytoplasmic features were vacuoles (some autophagic), myelin figures and ferritin deposits (fig. 1b). Nuclear abnormalities observed included widened nuclear pores, nuclear membrane whorls, abnormal heterochromatin distribution, incomplete nuclear division, karyorrhexis and nuclear vacuoles, and absence of the nuclear membrane (fig. 1b).

The patient's granulocytes contained intranuclear vacuoles, fewer than normal numbers of granules, nuclear membrane whorls, iron-laden mitochondria and widened nuclear pores (fig. 2b). An outstanding feature was the presence of the cisternae (either perinuclear or peripheral) in 177 of 200 (88.5%) granulocytes examined. Several platelets were seen, and showed myelin forms and cytoplasmic cisternae (fig. 1c).

The erythrocytes of the patient's relatives were normal except for a single cell in over 1,000 examined from his father. This cell had an unusual cytoplasmic vacuole.

All erythrocyte precursor cells from the bone marrow showed the nuclear and cytoplasmic abnormalities outlined above plus intranuclear ferritin, pyknotic nuclei, asynchrony in the development of nuclei within bi- or multi-nucleated cells and cytoplasm within the perinuclear space (fig. 3a). Polyribosomes and microtubules were present in many cells.

VBL-treated erythrocytes from a healthy donor showed vacuoles, whorls at the cell membrane, and peripheral cisternae in 5 /





Fig. 3. Transmission electron microscopy. a Bone marrow erythroblast showing cytoplasm in the perinuclear space (1), abnormal vacuoles (2), and peripheral cisterna (3). × 15,700. b VBL-treated erythrocyte from healthy donor showing cytoplasmic vacuole (1), peripheral cisterna (2), and cell membrane whorl (3). × 33,500

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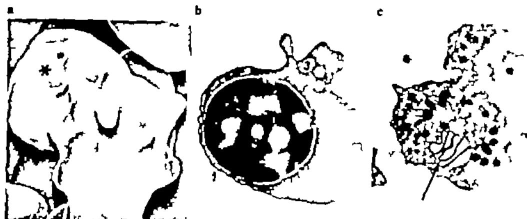


Fig. 1. Scanning and transmission electron microscopy. a Bizarre erythrocyte morphology including membrane pits and depressions (*). × 4,500. b Peripheral blood erythroblast showing peripheral cisterna (1), widened nuclear pores (2), and cytoplasmic vacuoles (3). × 5,000. c Platelet showing vacuoles and stacks of cytoplasmic cisternae (arrow). × 9,600.



Fig. 2. Transmission electron microscopy. a Fingerlike projection containing the cisternae (arrow). × 16,400. b Granulocytes showing peripheral cisternae (1), perinuclear cisternae (2), widened nuclear pores (3), and a phagocytic vacuole (4). × 5,500.

Transmission electron microscopy of the peripheral blood revealed that 52.7% of the 1,036 erythrocytes examined contained peripheral cisternae. All nucleated RBC in circulation also contained the cisternae. Fingerlike projections from the erythrocytes often contained the cisternae (fig. 2a). Layers of cisternae, internal cisternae and cisternae circumscribing entire cells could be found. Other cytoplasmic features were vacuoles (some autophagic), myelin figures and ferritin deposits (fig. 1b). Nuclear abnormalities observed included widened nuclear pores, nuclear membrane whorls, abnormal heterochromatin distribution, incomplete nuclear division, karyorrhexis and nuclear vacuoles, and absence of the nuclear membrane (fig. 1b).

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VBL treated erythrocytes from a healthy donor showed vacuoles, whorls at the cell membrane, and peripheral cisternae in 5 /



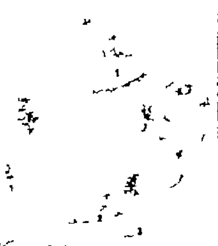


Fig. 3. Transmission electron microscopy. **a** Bone marrow erythroblast showing cytoplasm in the perinuclear space (1), abnormal vacuoles (2), and peripheral cisterna (3). × 15,700. **b** VBL-treated erythrocyte from healthy donor showing cytoplasmic vacuole (1), peripheral cisterna (2), and cell membrane whorl (3). × 33,500

of the 500 cells viewed (fig 3b) VBL did not alter the morphology or the proportion of erythrocytes displaying abnormal features in the patient.

Discussion

Ultrastructural studies of this patient have revealed several interesting differences from patients with typical CDA type II We found that 52.7% of our patient's erythrocytes contained the peripheral cisternae or double membranes most diagnostic of CDA II In contrast, *Hug et al* [9] determined that only 1–2% of the cells from their patient were so affected.

Scanning electron microscopy of the patient's cells also showed erythrocyte surface abnormalities of a type not previously described [12]

A striking feature of this case was the involvement of the other blood cells The nuclear and cytoplasmic defects seen in his erythrocytes could be demonstrated in the granulocytes and platelets. Only a minority (11.5%) of the granulocytes were devoid of marked ultrastructural abnormalities. There have been few previous reports of granulocyte abnormalities in CDA. Furthermore the abnormalities in these cases have been far less striking [19] than in the present case Our morphological evidence of abnormalities in the granulocytes and platelets supports the view of *Boivin* [1] that the primary genetic defect in CDA is at the stem cell level.

All of the erythrocyte precursor cells we examined from our patient showed ultrastructural evidence of dyserythropoiesis, including nuclear abnormalities of the kinds previously reported in all three types of CDA Other authors have reported that not all erythroid precursor cells in their cases were abnormal [2, 8 9 13 21] This seems surprising, because all cells within a given cell population should be affected when the defect is genetic, unless some cells are able to overcome the genetic damage [6]

We conducted two experiments with VBL, an agent known to act on the red cell membrane and the spectrin-containing microfilamentous network underlying the membrane [10] We found it produced a morphological picture strikingly similar to that of the patient in 5% of the red cells obtained from a healthy donor This suggests that some of the ultrastructural features of the patient's disease may be the result of a primary defect in the cell membrane, as we have previously postulated [14] A cell membrane defect could give rise to the nuclear and cytoplasmic features of dyserythropoiesis [11 20] An alternative explanation by *Lewis and Frisch* [12] is that the abnormalities in the membrane are secondary to a nuclear membrane – nuclear pore defect causing abnormal DNA and RNA synthesis.

Although the CDAs are rare conditions, and our patient is an exceptional case, detailed investigations of such patients may contribute to our understanding of dyserythropoiesis in general

Acknowledgements

The authors wish to thank Prof *R Rodda* of the Pathology Department, University of Tasmania, for making available the electron microscope and Mrs. *Sandra Petrie* for typing the manuscript.

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Catherine Dewar, PhD,
Department of Medicine,
University of Tasmania,
Box 252-C, GPO, Hobart,
Tasmania 7001 (Australia)

Book Reviews

C G Geary (ed.)
Aplastic Anaemia.
Baillière Tindall, London 1979
249 pp 39 fig 32 tab 2 cpl £ 12.50
ISBN 0-7020-0698-X

This book *provides an in-depth analysis of* various syndromes characterized by deficient 'stem cell proliferation. The topic has been divided into ten chapters which were written by the editor as well as by a number of invited experts from the United Kingdom, *Norway Germany* and the United States An introduction on pathophysiology (*C G Geary* and *N G Testa*) is followed by discussions of drug-induced bone marrow aplasia (*H B Benestad*), clinical features (*D M Williams*) laboratory *studies (H Heimpel), dysery*thropoiesis (*S M Lewis*) and treatment, including bone marrow transplanation (*E. C Gordon-Smith*) The last three chapters cover special aspects of aplastic anemia in childhood (*D I K Evans*), red cell aplasia (*C G Geary*) and the aplasia-leukemia syndrome (*G R Milner* and *C G Geary*). The book has not been written primarily for the expert. both experimental procedures (e g. cultures of hematopoietic progenitor cells) and therapeutic management are presented in a way which may be understood by the clinician as well *as by those who work primarily in the laboratory* Each chapter contains a bibliography covering historical developments as well as recent research. The general presentation is excellent, including a number of informative figures and a carefully accumulated subject index. The book can be recommended as a valuable source of information concerning ll *aspects of bone marrow failure.*

E. A. Beck, Berne

A Helle
Zytochemische Diagnostik in der klinischen Hämatologie.
Thieme, Stuttgart 1979
VIII + 158 pp., 73 fig., 41 tab *DM 30 –*
ISBN 3-13-577301 9

The title of this monograph is somewhat misleading cytochemical methods discussed include alcaline phosphatase, chloroacetate esterase, acid phosphatase, adenosine 5-triphosphatase, leucine aminopeptidase, β-glucuronidase, and PAS staining which are all described in detail, but not the more common procedures. *Furthermore*, 'clinical hematology in the author's experience, focuses on the diagnostic approach to myeloproliferative disorders, malignant lymphomas and the various leukemias. Obviously the author studied himself a vast number of these disorders and attempted to establish diagnostic criteria on the basis of the *mentioned procedures. The* need to rediscuss general features of hematologic neoplasias in this context is questionable. In view of the large number of personal observations it is somewhat difficult to gain insight into the most relevant data. At *the end of the book one finds 222 references cited* in the text, but no subject index. On the whole, the monograph does not qualify as a review but it certainly contains many personal observations which merit the attention of hematologists interested in the histochemical differentiation of the hematological abnormalities discussed.

E. A. Beck, *Berne*

G A McDonald T C Dodds and B Cruickshank (eds.)
Atlas der Hämatologie;
3 überarbeitete und *erweiterte Aufl.*
Thieme, Stuttgart 1979
X + 305 pp 408 fig. (mostly color photographs) DM 98 –
ISBN 3-13-374303-1

The German translation of the highly success*ful Atlas of Haematology' first published in* 1965 corresponds to the fourth English edition, published in 1977 The morphology of normal and abnormal blood cells and hemopoietic precursors is illustrated by e cellent color microphotographs. A new chapter on transmission and scanning electron microscopy of blood cells ha been added. *Numerous illustrations* refer to the histology of bone marrow lymph nodes, as well as to organ *manifestations of hematologic disorders. A special* chapter has been devoted to parasites encountered in the blood and *their life cycles. Although this*

recent edition, as its predecessors, concentrates on purely morphological evaluation of hematological disorders one would wish to have the modern concept on stem cell differentiation included in the next edition. The table on normal reference values would also benefit from revision (the upper limit of platelet counts 400,000 platelets/μl – being certainly too high). Furthermore, methods for differentiating normal and abnormal lymphocytes might be more detailed The Atlas is still most useful book, especially for medical students and laboratory technicians. Hematologists and clinical pathologists will need it as standard reference for diagnosing some of the rare disorders which are well illustrated in this outstanding book.

E. A. Beck, Berne

Blood Cells and Vessel Walls: Functional Interactions
Ciba Foundation Symposium 71 (new series)
Excerpta Medica, Amsterdam 1980
357 pp US$ 43.50
ISBN 0-444-90114-4

This symposium, held in March 1979 was organized in honor of *J. L. Gowans* who is well known among hematologists and immunologists for his pioneering work on lymphocyte recirculation. Dr Gowans chaired the Meeting. Accordingly the content of the Symposium Proceedings reflect the primordial interests of its organizer lymphocyte kinetics, including studies of their recirculation in adult and fetal life, cell adherence to the vessel wall (mainly lymphocytes and granulocytes), with special emphasis on the role of 'high endothelial venules The presentations are, at least in part, highly technical. In this respect, the book can hardly be recommended as review for the less initiated

The book starts off with summary on the ultrastructure of the bone marrow 'microenvironment' by *L. Weiss*. A further morphological study by *W. van E. uk* presents the 'microenvironment' of the lymph node and thymus. A third, predominantly ultrastructural study by *M. Simionescu* attempts to classify various types of microvascular endothelium A further chapter on blood platelets considers various stimuli which are possibly capable of platelet activation *in vivo (G. V. R. Born)*. The recent development in studies of platelet and vascular arachidonic acid metabolism is discussed by *J. R. Vane and S. Moncada*. *J. L. Gowans and H. W. Steer* review the discovery and implications of lymphocyte recirculation. *W. Trevella and B. Morris* consider particular aspects of the lymphoid apparatus in the sheep together with *R. N. P. Cahill et al.* who particularly discuss lymphocyte recirculation in the sheep fetus. The lymphocyte traffic through lymph nodes in experimental cell shutdown has been studied by *I. McDonnell et al.* Skin painting with oxazolone was being used as model for studies of delayed hypersensitivity *(J. G. Hall)*. Three further chapters are devoted to particular properties of high-walled endothelium in postcapillary venules of lymph nodes *(P. Andrews et al., J. J. Woodruff et al., E. C. Butcher et al.)* A particularly interesting presentation on heterogeneity of macrophages with respect to their interaction with lymphocytes *(J. H. Humphrey)* is followed by two reports on granulocyte chemotaxis *(S. H. Zigmond)* and adhesion *(P. D. Richardson)*. *I. L. Weissman and L. Weiss*, finally summarize the main results of the symposium and formulate some perspectives.

I feel that it is necessary to indicate the content of this volume. The far-reaching title might be somewhat misleading: interactions of red cells with vessel walls, both healthy or diseased, are barely mentioned Also, secretory functions of the vascular endothelium (with the exception of prostacyclin formation) have been largely neglected This is no serious criticism in view of the most stimulating information which may be found not only in the formal presentations but particularly in the discussion

E. A. Beck, Berne

A. Polliack
Normal, transformed and leukemic leukocytes. A scanning electron microscopy atlas.
Springer Berlin 1977
140 pp 236 fig DM 86.–/US $ 39.60
ISBN 3-540-08376-6

Surface properties of normal and leukemic leukocytes have been investigated by various techniques during the past decade. This monograph summarizes morphological features which are obtained by scanning electron microscopy (SEM). An introductory part describes current methods of preparation and cell fixation, as well as possible

artifacts. Numerous illustrations of SEM photographs illustrate normal human granulocytes, monocytes and lymphocytes. Factors which may affect surface properties of lymphocytes are critically discussed. Labeling studies on lymphocytes, as well as miscellaneous factors which influence surface properties of mammalian cells, are subsequently described. Further chapters discuss and illustrate mitogen-transformed lymphocytes, cultured cell lines and, finally surface properties of leukemic cells. Although the diagnostic usefulness of SEM is still limited this atlas serves its purpose of introducing the non-specialized hematologist into the possibilities of investigating surface properties of leukocytes. The quality of photographs and the general presentation are outstanding and justify the relatively high price of this book.

E. A. Beck, Berne

W. C. Maslow et al. (eds.)
Practical Diagnosis: Hematologic Disease
Houghton Mifflin, Boston 1980
584 pp $ 20.00
ISBN 0-89289-203-X

Das sehr handliche in Kunststoff gebundene Buch ist ein ausgezeichneter und hervorragend klar geschriebener Leitfaden über den Untersuchungsgang bei hämatologischen Erkrankungen. Differentialdiagnostische Erwägungen werden ausführlich besprochen. Die Laboratoriumsuntersuchungen werden im Prinzip geschildert und die entsprechenden Literaturhinweise erteilt. Die Abbildungen (schwarzweiss) sind ausserordentlich instruktiv. Das englisch geschriebene Buch ist uneingeschränkt für die Praxis und als Lehrbuch zu empfehlen.

H. Braunsteiner, Innsbruck

Original Papers

Acta haemat. 64: 61–64 (1980)

Satellitism of Platelets to Monocytes

A. M. Cohen, U. H. Lewinski, B. Klein and M. Djaldetti[1]

Department of Medicine 'B' and Electron Microscopy Unit, Hasharon Hospital, Petah Tiqva, and Tel Aviv University Medical School, Tel Aviv

Key Words. Chronic lymphocytic leukaemia · Myeloproliferative disorders · Monocytes · Platelet satellitism · Polycythaemia vera

Abstract. Satellitism of platelets to monocytes was observed in 3 patients suffering from polycythaemia vera, a myeloproliferative disorder and chronic lymphocytic leukaemia. This phenomenon occurred on cells in the buffy coat obtained from venous blood anticoagulated with heparin. The existence of platelet satellitism was reported to occur mainly to polymorphonuclears, but the present observation, as well as a previous report from our department, indicated that it may also exist to monocytes. Satellitism was achieved either by adherence of the platelets to monocytes, giving the impression of rosette formation, or by fusion of platelet pseudopodia with the monocyte membrane. The importance of this phenomenon in the evaluation of the platelet count using automatic instruments is discussed.

Platelet satellitism to peripheral blood polymorphonuclears was first documented in 1963 [1–4]. This rare phenomenon was found to occur mainly in venous blood anticoagulated with ethylenediamine tetra-acetic acid (EDTA), and resulted in inaccurate platelet count using automatic equipment. *Djaldetti and Fishman* [5] observed satellitism of platelets to monocytes even when the anticoagulant used was heparin. In the following case reports 3 patients are described who manifested platelet satellitism to monocytes obtained from heparinized venous blood. The patients suffered from polycythaemia vera, chronic lymphocytic leukaemia, and a myeloproliferative disorder. The platelet adherence to monocytes, resembling rosette formation, was shown by transmission electron microscopy.

[1] Established Investigator of the Chief Scientist's Bureau, Ministry of Health, Israel.

Case Reports

Case 1

A 65-year-old woman was referred to the clinic because of polycythaemia. She suffered from pruritus after warm baths for the last few months

The blood pressure was 150/90 mm Hg, the liver and spleen were not palpable.

Laboratory Findings. Haemoglobin 16.4 g/dl haematocrit 58 reticulocytes 1.2%, and the leucocytes 23,100/mm³ The differential count was normal ESR was *0 mm in the 1st hour (Westergren)* Platelet count was 1,080 000/mm³ counted with a binocular microscope, and 920 000/mm³ counted with an automatic counter (Technicon Model 1A) Blood volume was 91 cm³/kg body weight, and red blood cell volume was 46 4 cm³/kg body weight (normal values 67 cm³/kg and 28 cm³/kg respectively)

Case 2

A 69-year-old woman *suffered from* weakness and pruritus. On physical examination the liver span was 18 cm and the spleen was palpable 7 cm below the costal margin. Blood pressure was 150/80 mm Hg Enlarged lymph nodes were not *found.*

The haemoglobin was 14 1 g/dl, haematocrit 45%, leucocytes 19,300/mm³ with a marked shift to the left. The platelet number was 170 000/mm³ by microscopic counting and 160,000/mm³ by automatic counting. Cytogenetic studies showed normal karyotype Several attempts to obtain bone marrow aspirate failed. The diagnosis of a myeloproliferative disorder was established.

Case 3

A 69-year-old woman, known to suffer from chronic lymphocytic leukaemia for 3 years, was referred to the clinic. She felt well and was in a good general condition Enlarged and firm lymph nodes measuring 2–3 cm were palpated in the axillae and inguinal regions. The spleen was palpable 3 cm below the costal margin with hard consistency Haemoglobin was 12.5 g/dl, haematocrit 37%, white blood cells 23,000/mm³ with 84 / lymphocytes the platelet count was 185,000/mm by microscopic and 141,000/mm by automatic counting.

Electron Microscopy

Peripheral white blood cells were prepared from the buffy coat of 10 ml heparinized venous blood The cells were fixed in 1% glutaraldehyde in phosphate buffer pH 7 4 post fixed *in osmium tetroxide,* dehydrated in graded alcohols and embedded in Epon 812. Thin sections were cut with an LKB ultratome III and examined with a Philips 300 transmission electron microscope

Platelet satellitism appeared as rosette formation around the monocytes (fig. 1).



Fig. 1. Platelet satellitism to a monocyte 7,270

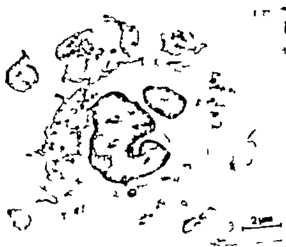


Fig. 2. Monocyte with phagocytotic vacuole. Around the cell are platelets showing satellitism. 8 600.

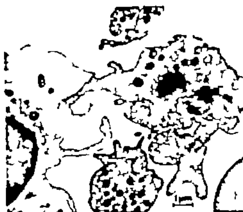


Fig. 3. Platelet pseudopodia in the process of fusing with monocytic pseudopodia. 17,300

The monocytes showed an increased number of vacuoles, partially because of platelet phagocytosis (fig. 2). In a few cases platelet particles were seen within the vacuoles. Satellitism was achieved by adhesion of platelet pseudopodia to the monocytes and subsequent lysis of their membranes (fig. 3).

Discussion

Platelet satellitism to polymorphonuclears was demonstrated in blood withdrawn with EDTA [1, 6–9]. This phenomenon was infrequently described in blood which was anticoagulated with oxalate, citrate or heparin [2].

Platelet satellitism is important because of possible false results of platelet counts by automatic counters. *Larson and Pierre* [10] showed that marked platelet satellitism modified white blood cell differential count when performed with the Technicon Hemalog D counter. In the 1st and 3rd patients described in the present report the number of platelets counted by automatic instruments was 85 and 70% of the respective number obtained with a light microscope.

In the patients described by us, as well as in the other cases [5, 9] the binding between platelets and monocytes resembled rosette formation. Monocytic pseudopodia were usually confluent with those of platelets, but some of the platelets underwent phagocytosis. *Kjeldsberg* [6] and *Kjeldsberg and Hershgold* [7] showed the binding between platelets and polymorphonuclears as partial or complete phagocytosis.

Plasma of the patients who showed platelet satellitism caused also platelet adsorption by leucocytes in healthy subjects. Plasma from a patient with hypogammaglobulinaemia caused satellitism of platelets to monocytes obtained from a healthy individual [5]. On the other hand, after incubation of the patient's platelets and monocytes with normal plasma the phenomenon of satellitism was not observed. These findings are in favour of the existence of a plasmatic factor which may be responsible for the satellitism. Indirect evidence which supports this assumption is the finding that heating of the patient's plasma to 70 °C or early incubation with concentrated platelets or leucocytes prevented satellitism.

The clinical significance of satellitism is as yet unknown, except for the explanation of spurious thrombocytopenia [7].

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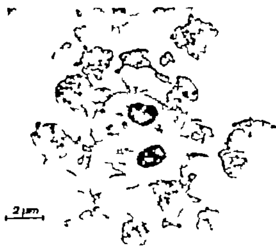


Fig. 1. Platelet satellitism to a monocyte. × 7,270.



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M. Djaldetti, Department of Medicine 'B' and Electron Microscopy Unit, Hasharon Hospital, Petah Tiqva (Israel)

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Micromegakaryocytes in Human Bone Marrow[1]

M. Wiesneth, H. Pflieger, B. Kubanek and H. Heimpel
Department of Internal Medicine, Division of Haematology and Oncology, University of Ulm, Ulm

Key Words. Micromegakaryocytes · Bone marrow · Pre-leukaemia

Abstract. Micromegakaryocytes (MMK) were defined morphologically by the cell area, nucleus form and cytoplasmic structure. Bone marrow smears of 7,156 patients were retrospectively analyzed. MMK were found most frequently and abundantly in acute non-lymphatic leukaemia, chronic myeloid leukaemia and pre-leukaemia. The presence of more than 10% MMK in the megakaryocyte population suggests a pre-leukaemic condition or non-lymphatic leukaemia. The platelet production of MMK is probably quantitatively normal although a functional defect is suspected.

Introduction

Megakaryocytes (MK), the largest human bone marrow cells, possess special morphological characteristics. During the maturation process they undergo endomitotic division and become polyploid and large in size [1, 15]. In some haematological diseases, the MK show typical morphological abnormalities: hyperlobulated nuclei occur in pernicious anaemia and especially large MK are found in polycythaemia vera. *Trautmann* [18] and later others [1, 5, 12] described extremely small and symmetrical MK in chronic myeloid leukaemia. In contrast to normal MK, these cells are termed micromegakaryocytes (MMK).

In our study, MMK were exactly defined under the light microscope. In order to elucidate the importance of the presence of MMK, bone marrow smears of 7,156 patients from Ulm University Hospital were retrospectively examined.

Material and Methods

Patient. Bone marrow smears of 7,156 patients were examined for the presence of abnormally small MK. Slides from 211 patients were selected for detailed cell size determination of MK as described below.

Bone marrow was aspirated from the anaesthetized iliac crest with a 20-ml syringe containing

[1] Supported by 'Deutsche Forschungsgemeinschaft' SFB 112.

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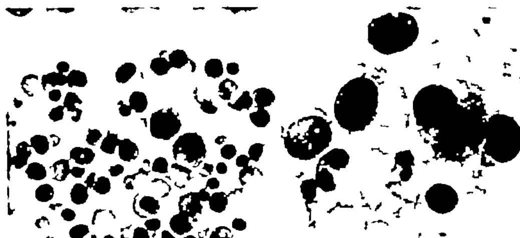


Fig. 2, 3. Micromegakaryocytes

MMK were found (fig. 2, 3). The details of these 1 4 patients with MMK are listed in table I. The average age was 55 years. Sex distribution was 58 females and 42 males. 53 of the patients had acute or chronic leukaemia. In 23 the diagnosis of a pre-leukaemic state had been made owing to various haematological abnormalities. No case of lymphatic leukaemia was found to possess MMK.

The number of MMK was expressed as a percentage of 100 thrombopoietic cells. The average percentage of MMK was far above 10 in manifest myeloid leukaemias and pre-leukaemic states. All other diseases, including acute leukaemia in remission revealed less than 10 MMK (fig. 4).

Of particular interest was the presence of MMK in the pre-leukaemic states. In 28 patients with pre-leukaemia, an average of 31 MMK was found. The tentative diagnosis of pre-leukaemia was supported by disturbances in production and or maturation of all haemopoietic cell systems [4 7 13 17]. The majority of these patients were

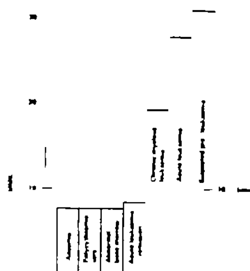


Fig. 4. Average MMK count in different diseases.

followed up at least 2 years or till death (table II). 10 of the 28 patients later developed leukaemia (6 acute myeloid leukaemia, 2 acute undifferentiated leukaemia 2 acute

0.5 ml anticoagulant solution. The bone marrow particles were placed on slides, fixed in methanol and stained according to Pappenheim.

Morphology and size of MK MK were classified morphologically according to their characteristic staining affinity at three stages of maturation, i.e. megakaryoblast promegakaryocyte and megakaryocyte [2]

Cell size determination was performed using a Leitz microprojector The well-defined cells with intact cell membranes were projected on white paper cut out, and weighed on an analytical balance (Mettler) Using the weight of the sample, the area of the paper was calculated and from this the cell area in square micrometers was derived. Uniformity of the paper weight was tested by tracing, cutting and weighing 50 fixed figures of five different sizes. The standard deviation of all 250 tracings was 0.97%. There was a strict correlation between paper weight and paper area ($r = +0.99$ $p < 0.001$) Applying a fixed magnification of $\times$ 2,500, the relation of paper weight to cell area was.

1 mg of paper corresponded to 1.55 μm cell area.

Results

MMK were defined using data reported in the literature as well as our own results. According to *Albrecht and Fülle* [1] and *Franzen et al* [5] in chronic myeloid leukaemia a large group of MK shows cell surface areas below 800 μm^2 The cytologic appearance of 1 or 2 round nuclei in these cells with normal mature cytoplasm [18] is in agreement with the observation of diploid DNA values of MK in patients with chronic myeloid leukaemia [12] We studied MK in 4 healthy persons and in 4 patients with haematological diseases (acute myeloid leukaemia, acute erythroleukaemia, chronic myeloid leukaemia, pre-leukaemia) known to possess a large number of abnormally small MK. For determining the exact cell size, 100 MK from each of them were measured (fig 1) The following definition of MMK was established

(1) the cell area must be smaller than 800 μm^2 [1 5]

(2) MMK must contain 1 or 2 small round and chromatin-dense nuclei [12]

(3) the cytoplasm should exhibit mature staining properties and a granular structure [18]

In 124 out of the 211 patients selected for detailed examination, one or more

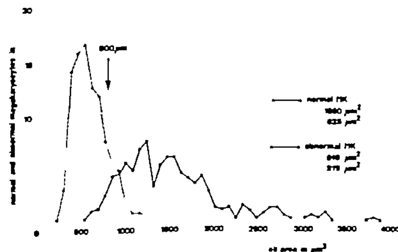


Fig. 1 Cell size distribution of 400 normal MK and of 400 abnormal MK in human bone marrow A statistically significant difference existed between the cell size of normal and abnormal MK ($p < 0.001$)





Fig. 2, 3. Micromegakaryocytes.

MMK were found (fig. 2, 3). The details of these 1 4 patients with MMK are listed in table I The average age was 55 years. Sex distribution was 58% females and 42% males. 53 of the patients had acute or chronic leukaemia. In 23% the diagnosis of a pre-leukaemic state had been made owing to various haematological abnormalities. No case of lymphatic leukaemia was found to possess MMK.

The number of MMK was expressed as a percentage of 100 thrombopoietic cells. The average percentage of MMK was far above 10% in manifest myeloid leukaemias and pre-leukaemic states. All other diseases, including acute leukaemia in remission, re ealed less than 10% MMK (fig. 4)

Of particular interest was the presence of MMK in the pre-leukaemic states. In 28 patients with pre leukaemia, an average of 31 MMK was found. The tentative diagnosis of pre-leukaemia was supported by disturbances in production and/or maturation of all haemopoetic cell systems [4 7 13 17] The majority of these patients were followed up at least _ years or till death (table II). 10 of the 28 patients later developed leukaemia (6 acute myeloid leukaemia, 2 acute undifferentiated leukaemia, 2 acute

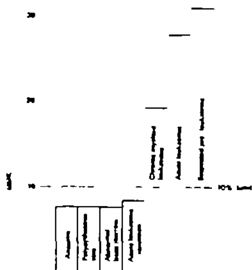


Fig. 4. Average MMK count in different diseases

Table I Diagnosis and MMK count in 124 patients

Diagnosis	Number of patients with MMK	MMK count, % mean	MMK count, % range	Number of patients with MMK > 10%
Chronic myeloid leukaemia	33	19	1–46	4
Acute leukaemia	6	28	5–85	20
Myeloid	16	30	5–85	1
Myelomonocytic	5	16	7 35	3
Erythroleukaemia		49	15–83	2
Undifferentiated	3	30	15–51	3
Acute leukaemia in remission	7	8	2–1	1
Myeloid	3	7	2 12	1
Undifferentiated	4	8	6–9	0
Suspected pre leukaemia	28	31	1–89	23
Polycythaemia vera	8	7	3–12	
Anaemia	7	7	1 15	
Iron deficiency	3	8	4–15	1
Megaloblastic	2	7	1 13	1
Infection	1	4		0
Renal	1	5		0
Nonspecific abnormal bone marrow	15	7	1 14	2
Carcinoma	5	8	5–11	1
Rheumatic arthritis	3	10	7–14	1
Pyelonephritis	3	4	2–6	0
Sarcoidosis	1	10		0
Tuberculosis	1	1		0
Mononucleosis	1	9		0
Erythema multiforme	1	5		0

erythroleukaemia) No relation existed between the MMK count and the interval up to the final diagnosis of leukaemia. Leukaemia appeared on average 7 7 months (range 1–32) after the detection of MMK. The average survival time was 3 1 months (range 0.5–10) after the diagnosis of leukaemia. 12 of 28 patients did not develop leukaemia, although they exhibited persistent haematological abnormalities typical of pre leukaemia. The average survival time of these 12 patients was 16 months (range 1–29) after the detection of MMK. 3 patients were still alive at the end of the study The causes of death in the other 9 patients were 5 cardiac failures, 2 cerebral haemorrhages, 1 septicaemia and 1 gastric carcinoma. The diagnosis of pre-leukaemia had to be changed in 6 out of the 28 patients, follow up studies revealing 1 case to be an iron deficiency an

Table II. Final diagnosis and average MMK count of 28 patients suspected to have pre-leukaemia

	Number of patients	MMK
Acute leukaemia	10	41
Suspected pre-leukaemia	12	29
No pre-leukaemia	6	6

aemia, 1 megaloblastic anaemia, 1 idiopathic thrombocytopenia, 1 pyelonephritis and 1 splenomegaly of unknown origin. 1 patient died of a heart attack. Only the patient with megaloblastic anaemia responding to vitamin B_{12} substitution had more than 10°/ of MMK.

The average percentages of MMK in the patients initially suspected to have pre-leukaemia are listed in table II. All patients with manifestations of leukaemia and with irreversible pancytopenia showed MMK counts greater than 10°/o. In contrast to this, all patients except 1 without further suspicion of pre-leukaemia had less than 10°/ MMK.

In 11 out of 58 non-leukaemic patients with MMK (pre-leukaemia, polycythaemia vera, anaemia, abnormal bone marrow), giant platelets were found in the peripheral blood. No statistically significant relation existed between the peripheral platelet count and the number of MMK in the bone marrow (r = -0.17).

Discussion

The morphology of MK in normal human subjects is extremely variable independent of their stage of maturity. This is true for cell size, number and shape of nuclei and nuclear/cytoplasmic ratio. The DNA content varies between 4 and 32 c with the majority of mature cells at 16 or 32 c [15]. The diagnostic significance of morphological aberrations of MK is therefore commonly regarded as less important than 'specific' abnormal morphological patterns in the erythroid and granulopoietic series. One exception is the presence of abnormally small mature MK in chronic myeloid leukaemia independently described in 1961 by *Trautmann* [18] and *Franzen et al.* [5] and later confirmed by others [1, 14, 17].

MMK can be recognized easily by light microscopy applying the following criteria: cell size, morphology of nucleus and maturity of cytoplasm. MMK are comparable to large promyelocytes with regard to cell size and can be identified without difficulty in routine work.

Previous studies from our laboratory established that a disturbance of polyploidization was responsible for the production of such abnormally small cells, called MMK [7, 16].

Casual observations of MMK in states other than CML prompted us to investigate the prevalence of MMK in a large number of unselected bone marrow smears collected in our institution.

No MMK was found in the marrow of normal individuals and only a few were found in marrow smears of some patients without haematological diseases. Thus, the number of MMK rather than their mere presence is of diagnostic significance. With few exceptions, more than 10° of MMK were seen only in myeloid leukaemias but not in malignant lymphomas. It is remarkable that in marrow smears of patients with acute non-lymphatic leukaemia in remission the MMK level was below the 10°/ limit.

The exact incidence of MMK in haemopoietic malignancies cannot be derived from

this study because the material was collected retrospectively from a large number of bone marrow specimens. However rough estimates showed that MMK were present in more than 20% of cases with chronic myeloid leukaemia acute myelomonocytic leukaemia and acute erythroleukaemia and between 10 and 20% in acute myeloid leukaemia and polycythaemia vera. Patients with chronic myeloproliferative diseases and distinct marrow fibrosis were not included, because this study was limited to the cytological analysis of aspirated bone marrow Within the non malignant conditions listed in table I MMK were noticed in less than 1% of all bone marrow smears examined

Of particular interest is the presence of a significant number of MMK in so-called pre-leukaemia Various prognostic factors predicting the onset of leukaemia in these patients have been reported [8 13] However differential diagnosis from refractory anaemia not terminating in leukaemia is still an unsolved problem The results of this study show that the presence of a significant number of MMK strongly indicates the evolution into a myeloid or myelomonocytic leukaemia. It has to be stressed that only 1 patient with megaloblastic anaemia responding to vitamin B_{12} in the group of no pre leukaemia in table II exhibited more than 10% MMK. The presence of more than 10% MMK in normoplastic or hypoplastic bone marrows of patients with unexplained pancytopenia therefore strongly suggests the existence of a true pre-leukaemic state The duration of the pre-leukaemic phase displays large variations so that a correlation with the MMK count can not be achieved. The development of MMK in the acute and chronic forms of non-lymphatic leukaemia, including pre-leukaemia and polycythaemia vera, is in agreement with the current concept of these leukaemias as the expression of a clonal disease arising from the pluripotent haemopoietic stem cell pool [3 6 10 19]

It is not known, whether the presence of large and abnormal platelets with ultrastructural abnormalities and functional defects as observed in myeloproliferative diseases [9 11 14] can be correlated to the presence of MMK No correlation exists between the number of MMK and the platelet count in the peripheral blood Since platelets are a product of cytoplasmic maturation and MMK show only abnormalities in the nuclear ploidization and not in the cytoplasmic maturation a quantitatively normal platelet production can be expected.

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M. Wienoeth, MD
Department of Internal Medicine,
Division of Haematology and Oncology
University of Ulm, Steinhovestrasse 9
D-7900 Ulm (FRG)

this study because the material was collected retrospectively from a large number of bone marrow specimens. However rough estimates showed that MMK were present in more than 20% of cases with chronic myeloid leukaemia acute myelomonocytic leukaemia and acute erythroleukaemia and between 10 and 20% in acute myeloid leukaemia and polycythaemia vera Patients with chronic myeloproliferative diseases and distinct marrow fibrosis were not included because this study was limited to the cytological analysis of aspirated bone marrow Within the non-malignant conditions listed in table I MMK were noticed in less than 1% of all bone marrow smears examined

Of particular interest is the presence of a significant number of MMK in so-called pre leukaemia. Various prognostic factors predicting the onset of leukaemia in these patients have been reported [8 13] However differential diagnosis from refractory anaemia not terminating in leukaemia is still an unsolved problem. The results of this study show that the presence of a significant number of MMK strongly indicates the evolution into a myeloid or myelomonocytic leukaemia. It has to be stressed that only 1 patient with megaloblastic anaemia responding to vitamin B_{12} in the group of no pre leukaemia in table II exhibited more than 10% MMK. The presence of more than 10% MMK in normoplastic or hypoplastic bone marrows of patients with unexplained pancytopenia therefore strongly suggests the existence of a true pre-leukaemic state. The duration of the pre-leukaemic phase displays large variations so that a correlation with the MMK count cannot be achieved. The development of MMK in the acute and chronic forms of non lymphatic leukaemia, including pre leukaemia and polycythaemia vera, is in agreement with the current concept of these leukaemias as the expression of a clonal disease arising from the pluripotent haemopoietic stem cell pool [3 6 10 19]

It is not known, whether the presence of large and abnormal platelets with ultrastructural abnormalities and functional defects as observed in myeloproliferative diseases [9 11 14] can be correlated to the presence of MMK No correlation exists between the number of MMK and the platelet count in the peripheral blood Since platelets are a product of cytoplasmic maturation and MMK show only abnormalities in the nuclear ploidization and not in the cytoplasmic maturation a quantitatively normal platelet production can be expected

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M. Wienneth, MD,
Department of Internal Medicine,
Division of Haematology and Oncology
University of Ulm, Steinhövelstrasse 9
D-7900 Ulm (FRG)

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Granulocyte Chalone Assayed *in vivo* in the Mouse

David J. D. Perrins, George Wiernik and William A. Jones

Research Institute, University of Oxford, The Churchill Hospital, Headington, Oxford, and Union International Research Centre, St. Albans, Herts.

Key Words. Assay · Chalone · Granulocyte

Abstract. An *in vivo* system has been developed for estimating the inhibitory activities of granulocyte chalone which overcomes the objection to the use of mixed proliferating target cells. It depends on the identification of labelled mature granulocytes in the peripheral blood of mice that received a radioactive precursor of DNA synthesis, with and without granulocyte extracts, several days previously.

Two extracts of bovine granulocytes were tested. The first demonstrated that maximum inhibition was achieved when it was given 2 h before the isotope, suggesting that its activity was short lived. It also failed to depress the labelling index of peripheral lymphocytes, thereby demonstrating tissue specificity. The second was used to show that the dose-response relationship was exponential.

When granulocyte extracts were prepared in sufficient quantity for initial clinical tests [*Rytömaa et al.*, 1976] it became possible to investigate an *in vivo* assay of material that did not depend on a mixed target cell population. Granulocyte extracts given to mice prior to the administration of tritiated thymidine (^{3}H TdR) depress the labelling index of leucocytes in the peripheral blood. This effect has been elaborated into an assay system.

The activity of granulocyte chalone has been demonstrated by *Rytömaa and Kiviniemi* [1967] by measuring the extent of inhibition of ^{3}H TdR into target cells in short term cultures. This basic technique is still in common use, although it has been modified in many ways. The principal drawback of the method, as appreciated by *Rytömaa and Kiviniemi* [1968] is that the tissue extracts are tested against mixed proliferating target cells. However, for short term assays, bone marrow is the only available source of normally proliferating precursor cells. It has long been appreciated that this type of test can only detect inhibitors acting at the G₁ + S portion of the cell cycle. In critical studies of the method, *Maurer et al.* [1976] concluded that it is no more than a non-specific screen for any

S-phase inhibitor requiring a supporting system for proof of true chalone activity For the screening of large numbers of chromatographic and other fractions it is still the only method which, however imperfectly satisfies the need for a relatively simple and rapid indicator system.

Schütt and Langen [1972] have pointed out that a simple sensitive test system, specific *in vitro*, is also lacking. We have attempted to satisfy this need by devising the technique described here, which seems to overcome the objections to the *in vitro* thymidine method. *Brecher et al* [1958] developed a technique that has been extensively used to study the behaviour of stem cells and their progeny They showed that mouse leucocytes could be labelled with single i p injections of H TdR, labelled lymphocytes appearing in the peripheral blood with a peak at 24 h and labelled neutrophils with a peak at 3 days. 5 days later both labelled granulocytes and lymphocytes were still seen but could not be detected at 8 days.

It is now generally accepted that ^{3}H-TdR is incorporated only into the nuclei of cells that are synthesizing DNA in preparation for mitosis and that in short-term studies of blood cells all such labelling is completed within an hour or so of the injection of isotope [*Spector and Coote* 1965] It seems reasonable to suppose that an inhibitor of incorporation into the DNA of leucocyte precursors should be detected, after a suitable interval, by estimating the relative numbers of labelled cells in the peripheral blood.

Preliminary experiments confirmed the well-known fact that white cell counts in mice are extremely variable both from day to day and from animal to animal, and are therefore unsuitable as an indicator of myelopoietic inhibition. They also showed that polymorphonuclear leucocytes comprise only ⁓5 of the total white cells. This relative paucity of granulocytes sometimes involved scanning may fields in order to find enough granulocytes to count. To overcome this problem the yield was increased by provoking a granulocytosis with *Escherichia coli* endotoxin. It was, however necessary to demonstrate that the granulocytes released from the reservoir compartments were labelled to the same extent as those from unstimulated mice and were true products of granulopoiesis and not just a trapped mature population.

Materials and Methods

Preparation and Examination of Blood Smears

6-week-old female DBA/2 mice, weighing approximately 20 g, were injected p with 20 μCi ^{3}H-TdR (5 0 Ci/mmol The Radiochemical Centre, Amersham). Smears of peripheral blood were prepared on acid-cleaned slides and fixed in absolute methanol Autoradiographs, set up using Ilford K_2 emulsion, were developed 6 week later stained (May-Grünwald-Giemsa) and examined by light microscopy (× 100 objective) and minimum of 100 granulocytes with segmented nuclei identified as labelled or unlabelled Lymphocytes encountered ere scored in simila manner

Granulocyt Extracts

Two preparations were used to demonstrate the inhibitory effect of granulocyte extracts on the incorporation of ^{3}H-TdR into the DNA of granulocyte precursors Bovine leucocytes were isolated by centrifugation after differential lysis of fresh whole blood The intact cells (and erythrocyte membranes) were extracted (4 × 1 h) with Hanks balanced salt solution and the combined extracts fractionated by ultrafiltration in Amicon hollow fibre and membrane systems The fraction of molecular size nominally between 500 and 10 daltons were isolated, lyophilized and subjected gel filtration on Sephadex G-25 The fraction el ing in the Ve/V range 1 8–2.3 was desalted

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Preparation and Examination of Blood Smears

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Granulocyte Extracts

T[illegible] preparations were used to demonstrat[e] the inhibitory effect of granulocyte extracts on the incorporation of ³H TdR into the DNA of granulocyte precursors. Bovine leucocytes were isolated by centrifugation after differential lysis of fresh whole blood. The intact cells (and erythrocyte membranes) were extracted (4 × 1 h) with Hank balanced salt solution and the combined extracts fractionated by ultrafiltration in Amicon hollow fibre and membrane systems. The fraction of molecular size nominally between 500 and 10,000 daltons were isolated, lyophilized and subjected to gel filtration on Sephadex G-25. The fraction eluting in the Ve/V[illegible] range 1.8–3.3 was desalted and

lyophilized. The sample of preparation A was obtained from the bulked extracts. In short term culture tests, using rat bone marrow 40% inhibition was observed at a dose level of 100 μg/ml. It was used to establish biological activity and determine the optimal interval between the administration of ^{3}H TdR and granulocyte extract.

Preparation B was obtained by a similar procedure, except that the cells were extracted for only 1 h. In the bone marrow test it appeared to be weakly stimulatory (10–15%) at 100 μg/ml. *Rytömaa and Kiviniemi* [1968] have already observed that, using their bone marrow assay the amount of chalone obtained in successive extractions of granulocytes apparently reaches a peak in the third extract. This is the result of a concomitant release of a stimulatory substance(s) which is extracted more readily than the chalone. Individual fractions from Sephadex G-25 columns corresponding to the Ve/Vo range 1.8–2.3 showed a peak of inhibitory activity reaching a maximum of 20% inhibition in the fraction Ve/Vo 2.05. The material which had been stored at –20 C, was made up at concentrations of 2.0, 0.2 and 0.02 mg in 0.2 ml of physiological saline.

Results

Leucocyte Counts

10 mice were used to establish the normal total and differential white blood cell counts, taken at the same time of day at a 4-day interval (table I). As expected, the total WBC counts are very variable but, in contrast, the differential counts are consistent and show that the granulocytes comprise only a quarter of the circulatory white

Table I. Mean total and differential white blood cell counts from 10 BDA/ mice

	Total WBC/mm³	Differential WBC count, %		
		granulocytes	lymphocytes	monocytes
Day 0				
x̄	11 631	25.3	69.7	5.0
SD	3 624	3.1	4.1	1.5
Day 4				
x̄	8 4[illegible]3	25.0	70.0	5.0
SD	1 620	3.3	4.9	1.3

Samples of tail blood were taken at the same time of day on days 0 and 4.

Table II. Mean differential white blood cell counts (± SEM) after intravenous E. coli endotoxin

Blood sampled at	Granulocytes, %	Lymphocytes, %	Monocytes,
	71 (± 8)	23 (± 9)	8 (± 1)
2 h	71 (± 12)	4 (± 13)	4 (± 3)
4 h	6[illegible] (± 11)	30 (± 8)	8 (± 4)
8 h	25 (± 3)	70 (± 5)	5 (± 1)
Controls (from table I)			

Mice were injected with 100 ng of endotoxin in 0.1 ml of saline into the tail vein. Smears of tail blood were prepared at intervals thereafter.

ls. 4 mice were injected with 100 ng of *E. coli* endotoxin in 0 1 ml of saline into the l vein. .. 4 and 8 h later smears of tail ood were prepared and the proportions of hite cell types determined (table II) It was hus possible to reverse the proportion of to lymphocytes, making the subsequent evaluation of the labelling in dices of granulocytes much easier

Labelling with H TdR

5 mice were injected with 20 µCi ³H TdR and blood samples taken at inter vals over 4 days. Autoradiographs of blood smears were prepared and examined as be fore As *Brecher et* al. [1958] had found, the labelling indices increased progressively with time (fig. 1). To determine whether cir culating granulocytes and those released by

Table III. Labelling indices of white blood cells of mice given *E. coli* endotoxin or saline

	Mice,	of total labelled (± SEM)	
		granulocytes	lymphocytes
Endotoxin	4	67 (± 3)	10 5 (± 3)
Saline controls	3	70 (± 10)	9 1 (± 2)

20 µCi ³H-TdR was injected p and 96 h later 100 ng of *E. coli* in 0 1 ml of saline was injected into tail veins. Smears of tail blood ere made 2 h later and autoradiographs developed after 6 eeks The number of mice, of the same strain as those used in the principal experiments, are undesirably (but not unacceptably) low due to circumstances beyond our control

Table IV Labelling indices of granulocytes and lymphocytes in the tail blood of DBA/2 mice sampled at 72 and 96 h after chalone or saline had been administered at the three time intervals before ³H TdR

Hours between saline or chalone and 20 µCi ³H-TdR	Mice,	labelled		Mice,	% labelled		Comparison of % labelled
		granulocytes	lymphocytes		granulocytes	lymphocytes	granulocytes
		Controls sampled at 72 h			*2.0 mg chalone/mouse sampled at 72 h*		p
2	5	75 4 (± 2 1)	11.. (± 1 7)	5	39 0 (± 3 8)	14 8 (± 4 0)	< 0 0005
8	5			5	37 0 (± 3 1)	14 0 (± 1.3)	
24	5	76 0 (± 3.0)	11 0 (± 1 2)	5	40 6 (± 8.3)	15 4 (± 3 4)	NS
		Controls sampled at 96 h			*2.0 mg chalone mouse sampled at 96 h*		
2	5	82.0 (± 2 0)	8 (± 0 8)	5	26.2 (± 6 0)	7.6 (± 0 5)	< 0 0005
8	5			5	36 0 (± 4 3)	14 0 (± 7 6)	
24	5	76 7 (± 4 4)	8 0 (± 1 7)	5	50 3 (± 10.8)	15 0 (± 3 5)	NS

Preparations too poorly labelled for reliable evaluation

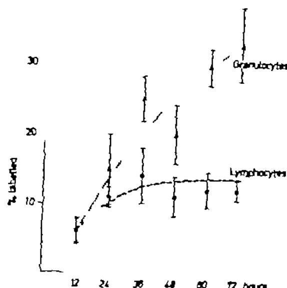


Fig. 1. Labelling indices (± SEM) of granulocytes and lymphocytes in the tail blood of 10 female DBA/2 mice at 12-hour intervals after i.p. injection of 20 μCi of ^{3}H TdR

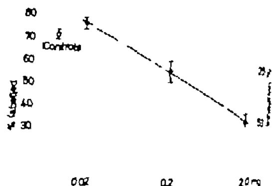


Fig. 2. Response to three dose levels of a granulocytic chalone preparation as shown by the inhibition of ^{3}H TdR incorporation into the DNA of granulocytes in peripheral blood.

endotoxin are labelled to the same extent by ^{3}H TdR, 20 μCi was injected i.p. into each of 4 mice and 96 h later 100 ng of *E. coli* endotoxin in 0.1 ml of saline was injected into the tail veins. 2 h later smears of tail blood were prepared and autoradiographs made up. 3 littermates were injected with saline to act as controls. The labelling indices are given in table III.

Effect of a Granulocyte Extract

Preparation A was used to establish biological activity and determine the optimal interval between the administration of ^{3}H TdR and granulocyte extract. 2.0 mg in 0.2 ml of 0.9% saline was injected into 18 mice, a similar number of controls receiving saline. Groups of 5 mice were each given 20 μCi of ^{3}H TdR, i.p. 2, 8 and 24 h after these injections. 72 h after the dose of label smears were made from tail blood. 24 h later (i.e. 96 h after the isotope injections) the mice were exsanguinated by cardiac puncture under ether anaesthesia and smears made. Autoradiographic data are given in table IV. When chalone had been given 2 h before the isotope, statistically significant differences (unpaired t test) between the labelling of granulocytes in the treated and control groups were found in mice whose blood was sampled at both 72 and 96 h after administration of ^{3}H TdR.

Dose Response to a Granulocyte Extract

Using the same technique, preparation B was assayed at three dose levels.

The extract (2.0, 0.2 and 0.02 mg) was given to groups of 6 mice, 2 h before the isotope. *E. coli* endotoxin (100 ng) was given by the tail vein 96 h after the isotope injection. 2 h later smears of tail blood were made. The results are shown in figure 2. The points fall on a straight line when the percentage of labelled cells is plotted against the dose of extract on a logarithmic scale. A 50% inhibition of labelling was obtained when 2.0 mg of extract was given, falling to 23% at 0.2 mg. As the control values are within 10% of the mean of the smallest dose, there is no significant activity at this

concentration. An indication of tissue specificity of the product is provided by the mean percentage of labelled lymphocytes remaining consistently within 10–14 %.

Discussion

By giving granulocyte extracts to mice prior to the administration of ³H TdR it has been possible to depress the labelling index of leucocytes in the peripheral blood. The maximum depression was achieved when the extract was given 2 h before the isotope, which is in accord with the findings of *Rytömaa and Kiviniemi* [1968] who showed that ³H-TdR incorporation into DNA is evanescent, peaking at 2 h in short-term assay. It also supports other evidence that chalones are short lived [*Iversen*, 1976].

When the interval between the injections of chalone and ³H TdR was increased to 24 h and the blood sampled at 72 or 96 h, the degree of inhibition was not statistically significant. This finding is of particular interest as it infers that the extract does not permanently impair the functional state of the progenitor cells. It has been shown that the granulocyte extract had no influence on the labelling index of circulating lymphocytes, thus the specificity of the product has been demonstrated against another closely related tissue in the same experimental preparation.

In the dose-response experiment, since only three concentrations of extract were used, it is not possible to determine the type of response; but as the points fall on a straight line when plotted on a logarithmic scale it seems reasonable to suggest that it is exponential. A dose-effect relationship for a granulocyte extract was established by *Rytömaa and Kiviniemi* [1968] using a bone marrow bioassay system. They showed that the maximal effect on the labelled cells was close to 50% and the relationship took the form of a cumulative normal distribution. A similar conclusion was reached by *Balázs et al.* [1978] assaying crude granulocyte extracts against rat myeloid leukaemia cells in culture. On the other hand, *Paukovits* [1973] again testing bone marrow cells in culture, showed a sigmoid response to increasing concentrations of purified chalone.

It should be appreciated that the present *in vivo* test, like others so far described, does not differentiate between the presence of mitotic inhibitors and stimulators (antichalones) but can only measure the relative equilibrium of both effects. The test does comply with the requirements for a valid assay system for the granulocytic chalone as defined by *Maurer and Laerum* [1976] namely it must measure qualitatively and quantitatively the inhibition of myelopoietic proliferation by a suspected chalone fraction; it must prove that a suspected chalone fraction is not toxic and does not impair the functional state of the cells; it must demonstrate the reversibility of the inhibition of proliferation by the suspected chalone fraction and it must show that it is granulocyte specific but not species specific. Although not a rapid technique, it could be used as an accurate assay for batch testing until such time as pure granulocyte chalone can be produced.

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Dr David J D Perrins, Research Institute, The Churchill Hospital, Headington, Oxford (England)

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Behaviour of Serum $\beta 2$ Microglobulin and Acute Phase Reactant Proteins in Chronic Lymphocytic Leukaemia

A Multicentre Study[1]

B. Späti, J. A. Child, S. M. Kerruish and E. H. Cooper

Unit for Cancer Research, University of Leeds and Department of Haematology, The General Infirmary, Leeds

Key Words. S-$\beta 2$-Microglobulin · CLL

Abstract. Changes in serum-$\beta 2$-microglobulin ($\beta 2$-m) levels were measured in 52 patients with chronic lymphocytic leukaemia during periods of observation of 5–43 months. While the majority of patients had elevated levels (> 3 mg/l), higher values tended to be associated with more advanced disease as assessed clinically. Patients with nonprogressive, predominantly Rai stage 0 and I disease had persistently normal or near normal $\beta 2$-m levels, whereas patients with progressive, predominantly Rai stage II–IV disease, had higher levels often showing rapid changes. Sequential measurements of serum $\beta 2$-m show patterns of change that reflect and may anticipate the clinical progression of the disease.

Introduction

Staging systems for chronic lymphocytic leukaemia (CLL) have shown a good correlation between stage and survival [3, 15]. They suggest that some patients probably do not require or will not benefit from treatment. The questions of when and how to treat patients with more advanced disease remain problematical. There is need for additional objective information to confirm the largely clinical assessments that the disease is 'stable' or 'progressive'. Current protocols which seek to decide which form of therapy is likely to be most effective at a given stage of disease would be considerably enhanced by the addition of objective indicators of response or lack of response.

β_2-Microglobulin ($\beta 2$-m) is a constituent of the HLA molecule, and occurs on the surfaces of all cells except erythrocytes [2, 7]. The serum $\beta 2$-m level is frequently elevated in patients with lymphoproliferative disorders including CLL [1, 4–6, 17, 18]. In non-Hodgkin's lymphomas the levels of serum $\beta 2$-m show a correlation with both the

[1] Investigators or institutions also participating: Dr. *J. Stone*, Leeds General Infirmary, Leeds, England; Dr. *A. V. Simmons*, Chapel Allerton Hospital, Leeds, England; Prof. *R. L. Turner*, Bradford Royal Infirmary, Bradford, England; Dr. *G. R. Tudhope*, Ninewells Hospital, Dundee, Scotland; Prof. *D. Hollard*, Centre Hospitalier et Universitaire de Grenoble, Grenoble, Cédex, France.

extent and aggressiveness of the disease [1 5] Sequential measurements of serum β2-m in CLL suggest that a persistently raised level is a feature of more advanced or progressive disease [6]

The results of a more detailed study over longer periods of time are presented in this paper The evaluation of the usefulness of the biochemical indicator is made in the realisation that the time course of CLL may be greater than a decade and that any particular group of patients represents diverse stages and rates of evolution of the disease.

Patients and Methods

The general conclusions made in this paper have been based on a survey of 168 patients followed for periods of at least half a year up to 4 years, in all contributing 243 years of experience.

All patients with CLL under surveillance at the commencement of the study were entered. In the majority 126 out of 168, the first measurement of β2-m was, therefore, at some time after diagnosis in new cases, coincident with time of diagnosis. Thus, in 42 patients, serum β2-m levels were obtained which covered the complete range of clinical stages, prior to any form of treatment.

In 52 patients, 17 females and 35 males, detailed longitudinal studies were undertaken. 18 of these received no treatment. Their mean age was 64 years (range: 40–79). As serum β2-m levels are known to be elevated in patients with a reduced glomerular filtration rate [20], the serum creatinine was measured on several occasions in patients in the longitudinal study all were found to have levels < 115 μmol/l (1.30 mg/100 ml) except case 2 terminally Serum β2-m levels were measured serially during observation periods of 5–43 months.

Blood samples were taken during visits to the haematology clinics or during treatment in hospital and stored at −20 C until analysed. 34 patients were treated during the follow-up period. The treatment usually consisted of intermittent high dose chlorambucil or constant low dose chlorambucil and corticosteroids or corticosteroids alone. Decisions as to the treatment were made on clinical grounds and not influenced by knowledge of serum β2 m levels.

During the course of the study 13 patients died, the cause of death directly related to the disease. The serum β2-m levels in this subset of the population were measured 1 year and 6 months prior to death all these patients were receiving therapy at the time these measurements were made.

Radioimmunoassay

β2-m was measured by radioimmunoassay using the Phadebas β2-m micro test (Pharmacia Diagnostics, Uppsala, Sweden) The upper limit of normal was taken at 3.0 mg/l which is higher than the usually quoted 2.4 mg/l, derived from healthy blood donors. This value was taken since we were dealing with an older group of patients; an increase of serum β2-m level with age has been shown [9]

Acute Phase Reactant Proteins

The serum levels of C-reactive protein (CRP), α_1-acid glycoprotein (AGP), α_1-antitrypsin (ATT) and ceruloplasmin (CPL) were measured by radial immunodiffusion using antisera and standards obtained from Behringwerke, Marburg/Lahn, FRG. Patients were considered to have an elevated C RP if the level was > 10 mg/l the upper limits of normal of the other proteins are AGP 1.4 g/l, ATT 4.5 g/l and CPL 0.6 g/l. The glycosylation of AGP was examined by crossed immuno-affinoelectrophoresis using concanavalin A as the lectin in the first dimension gel, as described by *Bøg-Hansen et al.* [11].

Results

The distribution of serum β2 m in untreated patients staged according to the Rai classification [15] are shown in table I

In the patients who died, the median value of serum β2 m 1 year before death was 7.5 mg/l with a range of 2.9–11.8 mg/l, and at 6 months prior to death the median value was 5 4 mg/l with a range of 3.2–12.0 mg/l

Longitudinal Studies. 3 Cases Selected to Show Particular Patterns of Change in Serum β2-m

Case 1 (fig. 1) shows the characteristic relatively stable β2-m in an untreated patient with stage II CLL who has shown no evidence of disease progression during a prolonged period of investigation. The β2-m was either slightly elevated or within the normal range.

Case 2 (fig. 2) shows the evolution of serum β2 m in a patient with disease progressing from stage II to stage IV and death. The re-introduction of treatment on clinical grounds, when the levels of β2 m were considerably elevated, had little effect on these

Table I. Kruskal-Wallis 1-way Anova test for significance ($\chi^2 = 18.60$, $p = 0.0001$). Untreated CLL (serum β2-m, mg/l)

	Stage		
	0	I II	III IV
Number	21	13	8
Inter-quartile range	2.0 2.8	2.7 5.3	4.4 16.9
Median	2.6	3.0	7.5
95% non-parametric confidence limit	~.0, 2.8	2.6, 4.7	3.0, 24.2

Range used.

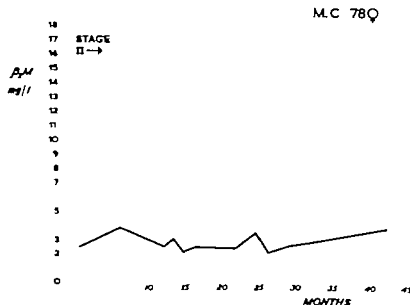


Fig. 1. Case 1. Stable β2-m levels in untreated stage II CLL.

Fig. 2. Case 2. Rising β2-m accompanying disease progression.

Fig. 3. Case 3. Reduction of β2-m following splenectomy and chemotherapy

levels. There was only slight clinical improvement. Serum creatinine was raised terminally.

Case 3 (fig. 3), a patient with stage II ... gross lymphadenopathy and massive splenomegaly, had raised serum β2-m levels initially with gradual decrease following splenectomy and treatment with intermittent high dose chlorambucil. Low platelet counts complicated management but these returned to normal eventually. Systemic symptoms and lymphadenopathy partially regressed during chlorambucil therapy but completely on steroids, and there is now no evidence of disease clinically. The changes in β2-m mirrored this response.

A schematic representation of the overall trend of the β2-m levels between the first and latest measurements in 18 untreated and 34 treated patients with CLL are shown in figures 4 and 5, respectively. Untreated patients with nonprogressive disease show a stable pattern. This is in contrast to the unstable pattern of the treated patients. Setting out the individual changes in relation to an overall duration of disease shows that marked instability is a feature of the initial response to therapy and also escape from control.

The distribution of the evolution of β2-m in the remainder of the population of treated CLL patients in the study is shown

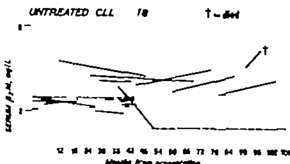


Fig. 4. Schematic trend of β2-m in untreated CLL.

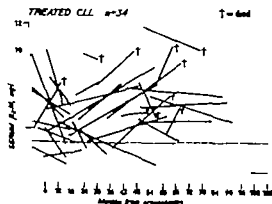


Fig. 5. Schematic trend of β2-m in treated CLL.

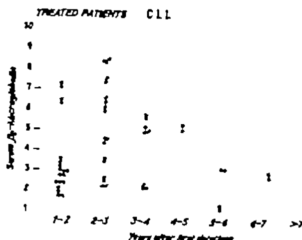


Fig. 6. Level of $\beta 2$ m in treated patients who are not included in figure 5 or table I.

in figure 6 All patients who survived 1 year and had any subsequent yearly points from the initial commencement of the study were included. Elevated levels of serum $\beta 2$ m are seen only in the initial years after detection, and it would appear that the long-term survivors are those that have persistently low values of serum $\beta 2$-m. In those patients who have disease Rai stages II–IV a wide range of serum $\beta 2$ m levels can be present. Whilst high levels > 10 mg/l did not appear to be sustained for more than 1–2 years, it is the rate of change that corresponded to a clinical improvement or progression of the disease

Acute Phase Reactant Proteins

The incidence of elevated C RP was 16 4% in samples taken during the summer months and 15 7% in those taken during the winter When the C RP was elevated it was almost always associated with some evidence of infection. At this time the other acute phase reactant proteins tended to rise coincidentally There was no coincidental rise of $\beta 2$ m during periods of overt infection. However a study of AGP in patients bearing large tumour loads (Rai stages III-IV) showed this could be elevated as high as 3.5 g/l (upper limit of normal 1 4 g/l) and that its elevation was dissociated from that of C RP AAT and CPL. This phenomenon was not encountered in patients with small tumour burdens. Lectin immuno-affinoelectrophoresis showed the AGP retained its normal glycosylation pattern in all stages of CLL.

Discussion

Vertical studies, notably in an *untreated* group of patients, confirm that there is a correlation between stage assessed clinically and serum $\beta 2$ m level.

There is a broad similarity between the findings in this report and those in non-Hodgkin s lymphomas where an incidence of raised serum $\beta 2$ m of 23% in stage I and II disease was found in comparison with 60% in stage III and IV disease [5]. Untreated non-Hodgkin s lymphoma patients with unfavourable histology tend to have higher levels than those with more favourable forms In active Hodgkin s disease the incidence of a raised $\beta 2$ m is comparable [1 5] Not surprisingly longitudinal studies of patients with CLL showed a generally more stable pattern of serum $\beta 2$-m than that seen in the lymphomas. The steady increases in serum $\beta 2$ m levels occurring over periods of several months was concomitant with progression of disease and appears to give a longer 'lead time than in NHL. Relatively rapid increases in serum $\beta 2$-m or high levels (> 10 mg/l) unaffected by treatment appear to indicate poor prognosis.

The origin of the increased serum $\beta 2$ m in CLL is uncertain. *Plesner et al* [14] have

demonstrated that the HLA-associated β2-m per lymphocyte is unchanged but that β2-m associated with other cell constituents probably the cell membrane, is re... they demonstrated that the serum β2-m is not complexed with glycoproteins. Massive rapid cell death in chemosensitive ... and lymphoma is not associated with transient elevations of serum β2-m [5, 10] contrasting with behaviour of other metabolites derived from cell death such as urate and polyamines [8]. Lymphocyte stimulation by mitogens *in vitro* is accompanied by marked increase of β2-m production [12] and most lymphoid cell lines in culture secrete β2-m into the medium [13]. One possibility is that transformed lymphocytes could be responsible for the increased serum β2-m, but this would imply a chronic stimulation of such cells being sustained for many years.

It was of interest that out of 21 patients with stage 0 disease, that is with lymphocytosis alone, only 3 had a level of β2-m greater than the upper limit of normal for elderly persons. This strongly argues against there being a simple relationship between the number of lymphocytes in the peripheral blood and the serum β2 m. Furthermore, there can be marked differences in the levels between one patient and another with apparently the same tumour load as judged clinically which supports the hypothesis that it may be a subset of the lymphocytes that control the serum β2-m level rather than the tumour mass as a whole. This hypothesis would then bring together the observed disturbances of β2-m production that are found in CLL, malignant lymphomas and multiple myeloma, where the common phenomenon could be the activity of a particular subset of the lymphocytes. Such a possibility has been suggested to account for raised serum β2-m in diseases such as rheumatoid disease, cirrhosis and Crohn's disease [19].

The measurements of APRPs indicate that β2-m levels are independent of the response of this family of proteins to infection in CLL patients. The cause of the dissociated rise of AGP is unknown; the evidence would seem to be against an extra-hepatic synthesis as proposed by *Gahmberg and Anderson* [16] but could reflect a local disturbance in the liver produced by lymphocytic infiltration.

There are many gaps in our knowledge of the function of β2-m and the homeostatic controls that are operating when raised serum β2-m levels occur in lymphoproliferative disorders. Nevertheless, this study has shown that the changes in serum β2-m levels may reflect disease activity in CLL and suggests that serial measurement can help in decision-making in the management of this disease.

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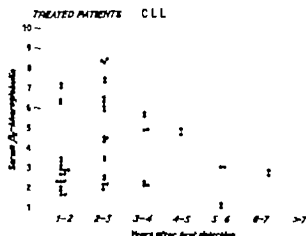


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Prof. E. H. Cooper The University of Leeds,
The Unit for Cancer Research
School of Medicine,
Leeds LS2 9NL (England)

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Serum β_2-Microglobulin, Serum Creatinine and Bone Marrow Plasma Cells in Benign and Malignant Monoclonal Gammopathy

Andreas Morell and Walter Riesen

Institute for Clinical and Experimental Cancer Research, University of Berne, Tiefenau Hospital, Berne

Key Words. Creatinine · β_2-Microglobulin · Monoclonal gammopathy benign, malignant · Plasma cells

Abstract. Serum β_2-microglobulin concentrations were determined in samples of 65 patients with benign or malignant monoclonal gammopathy. In the group of patients suffering from multiple myeloma or Waldenström's macroglobulinemia the mean β_2-microglobulin level was significantly higher than in the group with benign monoclonal gammopathy. Values above 3 mg/l were highly indicative of malignant disease and observed in 50% of the myeloma patients. Serum creatinine levels were significantly correlated to β_2-microglobulin levels. However, mean creatinine concentrations did not significantly differ between the two groups of patients. Plasma cells and lymphoplasmocellular elements containing cytoplasmic immunoglobulin were counted in bone marrow samples of all patients. The counts, expressed in percent of nucleated bone marrow cells, allowed a good discrimination between the benign and the malignant group of patients. Bone marrow from patients with multiple myeloma or macroglobulinemia contained more, from patients with benign monoclonal gammopathy less than 17% plasma cells. No significant correlation was noticed between the extent of this plasmocytic bone marrow infiltration and serum β_2-microglobulin or creatinine levels.

Introduction

Today reports on measurements of tumor associated antigens are often received with some scepticism, since too many of these tests have failed to fulfill the expectation of being useful cancer markers [13]. Nevertheless, studies on serological and other markers of malignant disease have to be continued with the hope that finally methods allowing for reliable and early detection of malignant tumors will be developed. One of the markers widely studied in this context is β_2-microglobulin (β_2m), a polypeptide with a molecular weight of 11,900 which is integrated into the structure of HLA antigens on the cytoplasmic membrane of all nucleated cells [20]. Increased serum concentra-

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Prof. E. H. Cooper The University of Leeds,
The Unit for Cancer Research
School of Medicine,
Leeds LS2 9NL (England)



Fig. 1. β_2m serum concentrations in patients with benign and malignant monoclonal gammopathy. Bars indicate geometric means ± 1 SD. The dotted area represents the normal range.

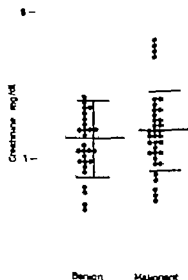


Fig. 2. Creatinine serum concentrations in patients with benign and malignant monoclonal gammopathy. Bars indicate geometric means ± 1 SD. The dotted area represents the normal range.

Serum Creatinine Levels

Mean serum levels in the two groups of patients were rather similar: 1.25 versus 1.37 mg/dl (fig. 2). This difference is not significant (t-statistics, $p \sim 0.37$). In the benign group 6 sera (22%) were at or above the upper normal limit compared to 10 sera (29%) in the group with malignant monoclonal gammopathy.

Correlation between Serum β_2m and Creatinine Concentrations

When serum β_2m and creatinine concentrations of all patients with monoclonal gammopathy were compared in a scatter diagram (fig. 3), a weak but statistically significant positive correlation was observed ($r = 0.39$, $p < 0.05$). Values for the group with malignant disease were somewhat better correlated ($r = 0.47$) than those of the group with benign monoclonal gammopathy ($r = 0.31$).

Bone Marrow Plasma Cells

The values for bone marrow plasma cells and lymphoplasmocellular elements containing cIg are given in figure 4 in percent of nucleated bone marrow cells. Mean values were 8% in the benign and 31 in the malignant group. This difference was highly significant (t-statistics, $p < 0.001$). The two groups of monoclonal gammopathy were reasonably well separated when a dividing point of 17% cIg positive bone marrow plasma cells was assumed (fig. 4). Only 5 bone marrow samples of the benign group

tions of β_2m are observed in cases with high cell proliferation rates in lymphoproliferative disorders such as monoclonal gammopathies or leukemias, and in certain other malignancies [1 2, 7 8, 11 12, 14 15 22–24] Serum concentrations also depend on the glomerular filtration of β_2m [3 4 27] and are thus affected in patients with impaired renal function. The differentiation whether an increased β_2m value is due to increased synthesis or rather to decreased glomerular filtration can be made by a parallel determination of serum creatinine [6 27]

In continuation of our earlier studies [18] we were interested in the value of serum β_2m determinations as an aid in distinguishing between benign monoclonal gammopathy and malignant gammopathies, such as multiple myeloma and Waldenström's macroglobulinemia. For this purpose we compared serum β_2m levels with the degree of bone marrow infiltration by plasma cells. Furthermore, we studied the interdependency of β_2m creatinine levels and bone marrow plasma cells in this clinical model.

Patients and Methods

Serum and bone marrow specimens of 63 patients with monoclonal gammopathy were studied. The patients were between 30 and 83 years old. Most of them were over 60 years old. Both sexes were about equally represented. Differentiation between benign and malignant monoclonal gammopathy was made by generally used diagnostic criteria [16, 26] 35 patients were hospitalized with multiple myeloma or Waldenström's macroglobulinemia, whereas in 28 patients a benign monoclonal gammopathy was diagnosed. Serum and bone marrow samples were obtained at the time of the diagnosis. Thus, the patients were not under chemotherapeutic treatment.

Serum concentrations of β_2m were determined by a solid phase radioimmunoassay (Phadebas, Pharmacia Diagnostics, Uppsala, Sweden). The normal range of 1.1–2.4 mg/l was established on sera from healthy blood donors. These concentrations agree with previous reports [2, 22].

Serum creatinine levels were measured using the Jaffé reaction, without deproteinization, in a fixed-time kinetic analysis on a Coulter Kem-O-Mat analyzer The normal range was 0.70–1.60 mg/dl.

Details on the determination of bone marrow plasma cells have previously been described [19]. Briefly cytocentrifuge slides were prepared from bone marrow samples, fixed in ethanol-acetic acid and stained with a FITC-labelled polyvalent anti-immunoglobulin reagent. The preparations were examined under a fluorescence microscope with incident illumination. Plasma cells and lymphoplasmocellular elements containing cytoplasmic immunoglobulin (cIg) as well as unstained nucleated bone marrow were counted. Values for cIg positive plasma cells were expressed in percent of nucleated bone marrow cells.

Statistics were calculated on a Hewlett Packard 97 using the programs SD-03A (curve fitting), CL1 18A, CL1 19A (t-statistics, t-distribution), and ST1-01A (basic statistics for two variables).

Results

Serum Values of β_2m

Most of the serum levels of the patients with benign monoclonal gammopathy fell within the normal range (fig 1) The mean serum concentration in this group was 1 48 mg/l which is comparable to the mean value of 1 60 mg/l in normal controls Six of the sera (21%) were at or above the upper normal limit of 2.40 mg/l.

In the group of the patients with malignant monoclonal gammopathy a mean serum concentration of 2.39 mg/l was observed (fig. 1). 17 or approximately 50% of these sera exceeded the upper normal limit. The difference between the means of the two groups was statistically significant (t-statistics, $p < 0\ 01$)

cause their management is different. In addition to the well-established criteria [16, 26] a number of differences exist which are important when distinguishing between the two groups of monoclonal gammopathy [5 9 17 18, 21, 25 26, 28]. It is in this context that determinations of serum β_2m have to be evaluated.

In 50% of the patients suffering from malignant monoclonal gammopathy serum β_2m levels clearly exceeded the upper normal limit of 2.4 mg/l (fig. 1). This agrees well with the results of previous investigations where between 39 and 74% of myeloma patients were reported to have increased serum β_2m levels [12, 14 15 22] Thus, a high β_2m concentration in a patient's serum is indicative of malignancy whereas a low level of β_2m does not exclude a malignant gammopathy Immunoglobulin class and light-chain type of the myeloma proteins did not have any influence on β_2m concentrations in previous studies [12, 24] nor did it in our material.

The benign group contained 6 sera (21%) with β_2m concentrations at or above 2.4 mg/l (fig. 1). Evidently the discriminant level has to be raised in order to obtain a separation between the two groups preferably to 3 mg/l – a figure which is used by most investigators in this field [1, 2, 7 8, 22, 23] This seems to be even more appropriate, since most patients were over 60 years old. It is well known that age and serum β_2m levels are positively correlated [10, 24] At a level of 3 mg/l only 1 patient of the benign group was 'misclassified' whereas 50% of the malignant group were still above this concentration. Serum creatinine concentrations differed much less between the two groups of patients and allowed no distinction. Very high values were found in 1 patient with benign monoclonal gammopathy who suffered from a severe staphylococcal pneumonia and in 4 myeloma patients who had marked Bence Jones proteinuria (fig. 2).

Serum levels of β_2m and creatinine were correlated in patients with monoclonal gammopathy The correlation between these two parameters, however was weaker than that reported by *Wibell et al.* [27] and by *Cassuto et al.* [6] in normal sera, and by *Kin et al* [14] in sera from tumor patients. Obviously monoclonal B cell proliferation acts primarily at the β_2m level. A rise of the creatinine concentration appears only when concomitant the kidney shows malfunction, and in turn this results in an even further increase of β_2m [27]

The results suggest an increase of serum β_2m in correlation with the tumor cell mass as already stated by *Cassuto et al.* [6] In multiple myeloma the tumor cell mass can be estimated by the staging system of *Durie and Salmon* [9] We thus assumed that β_2m levels were correlated to the extent of bone marrow infiltration by plasma cells. However no meaningful correlations were found. Likewise, no correlations were observed by *Shuster et al.* [22] between β_2m and clinical activity of plasma cell neoplasia, nor by *Fateh-Moghadam et al.* [12] between β_2m and serum concentrations of myeloma protein.

In order to evaluate the diagnostic value of β_2m determinations in monoclonal gammopathy we compared it with the degree of bone marrow infiltration by plasma cells and lymphoplasmocellular elements. It is known that this infiltration is much more pronounced in multiple myeloma and in Waldenström's macroglobulinemia than in benign monoclonal gammopathy In fact, this difference represents an important diagnostic criterion [16, 26] In general, the diagnosis of malignant gammopathy is vir

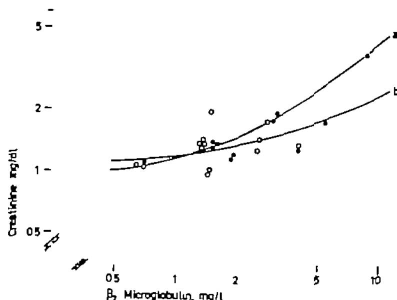


Fig. 3. Correlations between serum concentrations of β_2m and creatinine. O = Patients with benign monoclonal gammopath, y = 0.81 + 0.34 x, regression line a. ● = patients with malignant monoclonal gammopathy y = 1.08 + 0.13 x, regression line b.

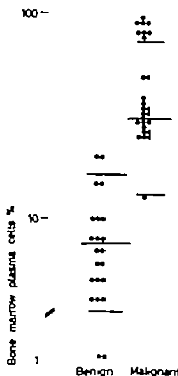


Fig. 4 Plasma cells and lymphoplasmocellular elements containing cIg in bone marrow of patients with benign or malignant monoclonal gammopathy Values are expressed in percent of nucleated bone marrow cells. Bars indicate geometric means ± 1 SD

were more densely infiltrated, whereas merely 6 samples of the patients with malignant disease contained less than 17% cIg positive bone marrow cells.

Correlations between Bone Marrow Plasma Cells Serum β_2m and Creatinine Levels

In our material no significant correlation was found between β_2m concentrations and the degree of bone marrow infiltration. Linear correlation coefficients of r = 0 15 in the benign, and of r = 0.22 in the malignant group were observed. Similarly no correlation was seen between bone marrow cells and serum creatinine levels. Linear correlation coefficients of r = 0 16 in the benign, and of r = –0 03 in the malignant group were noted.

Discussion

The differentiation between benign monoclonal gammopathy and multiple myeloma or related malignant disease is crucial be-

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Andreas Morell,
Institut for Clinical and
Experimental Cancer Research,
University of Berne, Tiefenau Hospital,
CH–3004 Berne (Switzerland)

tually certain when the proportion of bone marrow plasma cells exceeds 15–20% of the nucleated elements [26]. Figure 4 demonstrates that the two groups are optimally separated by a discriminant point of 17% bone marrow plasma cells. By using this discriminant point most sera (83%) could be assigned to the correct group. The diagnostic value of this test is higher than that of the β_2m determination. One has to object, however, that the method is much more complicated than a simple radioimmunoassay and that bone marrow is needed instead of serum. The 5 benign cases with more than 17% bone marrow plasma cells in figure 4 had serum β_2m levels between 1.3 and 2.8 mg/l, whereas only 1 of the 6 myeloma patients with modest bone marrow infiltration had high β_2m levels. Thus, it seems that a combination of both tests is of little help diagnostically.

In conclusion, the determination of the serum β_2m level in a patient with monoclonal gammopathy can provide valuable information on the malignancy of the disorder.

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Andreas Morell,
Institute for Clinical and
Experimental Cancer Research,
University of Berne, Tiefenau Hospital,
CH–3004 Berne (Switzerland)

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Nodular Lymphoma Eventuating into Lymphoplasmocytic Lymphoma with Monoclonal IgM/λ Cold Agglutinin and Bence-Jones Proteinuria

E Pascali A Pe‿oli M Melato and G Falconieri

Istituto di Clinica Medica Generale (Direttore· Prof. *F S Feruglio*) and Istituto di Anatomia e Istologia Patologica (Direttore· Prof *L. Giarelli*) University of Trieste, Trieste

Key Words. Cold agglutinins Lymphoplasmocytic lymphoma Monoclonal gammopathy Nodular lymphoma

Abstract. A case of nodular lymphocytic (centroblastic/centrocytic) lymphoma that developed within 5 months after the initial diagnosis of lymphoplasmocytic lymphoma is described. The change in the histopathological picture, consisting of the development of a functionally more differentiated B cell neoplasm, occurred in the abdominal lymph nodes while the original pattern persisted in the bone marrow liver and spleen The patient was also found to have a serum IgM/λ monoclonal immunoglobulin exhibiting cold agglutinin activity with specificity for the non I erythrocyte antigens.

Introduction

In the last few years a number of lymphoproliferative disorders, including most cases of chronic lymphocytic leukemia and of non-Hodgkin s malignant lymphomas, have been shown to be neoplasms of B cell lineage derivation These neoplasms are monoclonal with respect to the synthesis of their surface membrane and intracytoplasmic immunoglobulins The monoclonal immunoglobulin can also be secreted by the lymphoma cells in sufficient quantities for it to be detected in the serum by routine electrophoretic techniques. Some of the monoclonal immunoglobulins have been found to possess specific antibody activity directed against a wide variety of antigens, not infrequently self-components [17 24]

It may be assumed that the neoplastic event may occur at any of the stages through which normal B lymphocytes differentiate [22] Whether a particular cell type in the B cell lineage which undergoes neoplastic transformation can only proliferate being 'frozen at that stage or can still retain ability of further maturation/differentiation has not, however been well established

A number of investigators have reported the finding, based upon light microscopic studies of sequential biopsy and/or autopsy material of changes in cytological type and histological pattern within the non Hodgkin s lymphomas, particularly the nodular

lymphoma [5–7 19 25 28] It is uncertain, however whether the new histological pattern really represents the transformation of the nodular lymphoma or results from the proliferation of a separate malignant clone.

The present report describes a patient with nodular lymphocytic (centroblastic/centrocytic) lymphoma who developed rapidly (within 5 months after the initial diagnosis) a lymphoplasmocytic lymphoma associated with a serum IgM/λ monoclonal immunoglobulin exhibiting cold agglutinin activity with specificity for the 'non-I erythrocyte antigens.

Case Report

A 55-year-old man was admitted to the hospital in February 1979 with 3-month history of weakness, increasing fatigue, exertional dyspnea, low-grade fever epigastric pain with radiation to both the hypocondria, nontender mass in the left axillary region. Past and family history were unremarkable.

Physical examination disclosed generalized and symmetric lymphoadenopathy The lymph nodes were firm, nontender, freely moveable, discrete, 0 5–2 cm in diameter A greater one, approximately 5 cm in diameter was also appreciable in the left axillary region. Hepatomegaly nd splenomegaly (of 1 and 4 cm below the respective costal margins) were found

Pertinent laboratory data on admission included. ESR 40 mm in 1 h, Hb 10.3 g/dl, RBC 2×10^{12}/l, PCV 0 26, reticulocyte count 3%, WBC 7.4×10^{9}/l with differential of 69% neutrophils, 3% eosinophils, 1% monocytes, 27% lymphocytes, platelet count 220×10^{9}/l. An X-ray film of the chest was normal. Lymphangiography did not show enlargement of the iliac and para-aortic lymph nodes. Attempts to aspirate bone marrow failed repeatedly

Microscopical examination of biopsy specimen of left axillary lymph node revealed replacement of the normal structure by cellular infiltrate exhibiting nodular pattern in many areas but particularly in the paracortical medulla (fig. 1). The proliferation appeared to be constituted predominantly by centrocytes and centroblasts (fig. 2). The immunofluorescence studies performed on the biopsy specimen gave negative results.

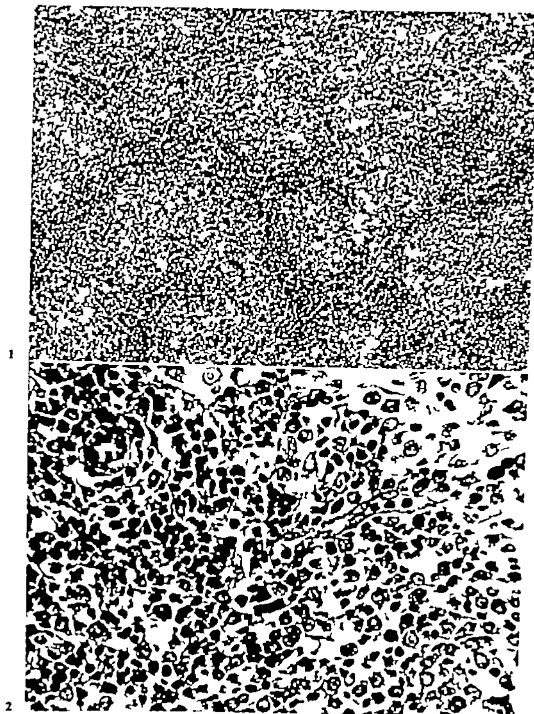
Rheumatoid serologic reactions were negative, cryoglobulins absent. Cold agglutinins were present to a titre of greater than 1:512. The direct antiglobulin test was positive. Total serum protein was 6 g/dl. Immunoglobulin levels were IgG 820 mg/dl, IgA 160 mg/dl, and IgM 450 mg dl by single radial immunodiffusion. Serum protein electrophoresis performed by cellulose acetate membrane (microzone) technique did not reveal any spike in the β or γ regions. The presence of serum M component could, however be suspected from the abnormal scanning diagram of another separation carried out by the same electrophoretic technique 2 weeks later (fig 3). At this time, immunoelectrophoresis was not performed. Urine was not investigated for the presence of Bence-Jones protein.

Despite 5 months of combination chemotherapy with CVP (cyclophosphamide, vincristine and prednisone), hepatosplenomegaly and generalized lymphoadenomegaly persisted and anemia worsened with increasing transfusion requirements.

On readmission to the hospital in July 1979 the hematological values were the following: Hb 5.6 g/dl, RBC 1.4×10^{12}/l, PCV 0 17 WBC 1.2×10^{9}/l, platelet count 100×10^{9}/l.

Serum protein electrophoresis performed on agarose gel according to *Johansson* [9] revealed the presence of monoclonal band. Immunoelectrophoresis [23], carried out in 1.5% agar gel in the same electrophoresis buffer identified the M component as IgM/λ. Electrophoresis of the concentrated urines (200 times by dialysis against polyethylene glycol in 18/32" Visking tubing), followed by immunoelectrophoresis with monospecific anti-free light chains sera, demonstrated Bence-Jones protein of λ type (fig 4).

Rheumatoid factor was negative, cryoglobulins absent The direct antiglobulin (Coombs') test was positive for complement A test for cold agglutinins using normal pooled group 0 adult (0I) erythrocytes was positive in titre of 1.4,000 at 4 °C with reactivity up to 25 °C. The same titre and thermal amplitude were found using normal



1

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Fig. 1. Left axillary lymph node biopsy. HE. × 16.

Fig. 2. Higher magnification of the same biopsy specimen as in figure 1. HE. × 100

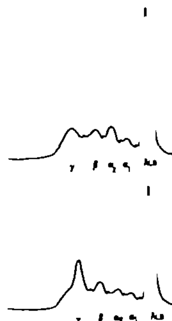


Fig. 3. Densitometric diagrams of the two serum protein electrophoresis on cellulose acetat membrane (microzone technique) performed t the first admission. Both electrophoresis were car ried out with serum samples separated at room temperature.

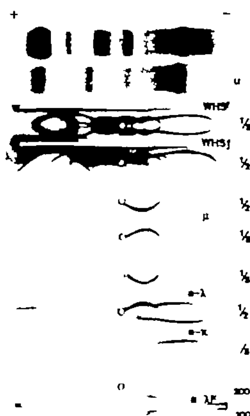


Fig. 4. Agarose-gel electrophoresis of the patient's serum ( ) and × 200 concentrated urine (u). The serum and urine monoclonal bands are clearly visible in the anodal side of the application slit. b Immunoelectrophoresis of the patient se rum ( ) and concentrated urine ( ) developed by applying different dilutions of the samples against an antiserum anti-normal whole human serum (a-WHS) and monospecific antisera to μ heavy chains (a-), K light chains (a-K), λ light chains (a-λ), and λ free light chains (a-λF)

pooled group 0 cord (0I) erythrocytes The cold agglutinin reaction was negative using papain- and neuraminidase-treated adult and cord erythrocytes

The patient's condition rapidly deteriorated and he died July 19 1979

The postmortem examination revealed enlargement of the liver (1,900 g) and the spleen (1,400 g). Widespread lymphoadenomegaly involving particularly mediastinal, para-aortic, iliac and mesenteric lymph nodes was observed.

A lymphoma exhibiting the same histological appearance as described in the biopsy specimen was seen in the femoral bone marrow liver and spleen. The microscopical examination of the autopsy specimens from the abdominal lymph nodes showed complete replacement of the structure by diffuse proliferation of cells exhibiting characteristics of plasma cells at different maturation stages. A great proportion of these cells, however was constituted by mature plasma cells, some of hich containing intracytoplasmic vacuoles (fig 5

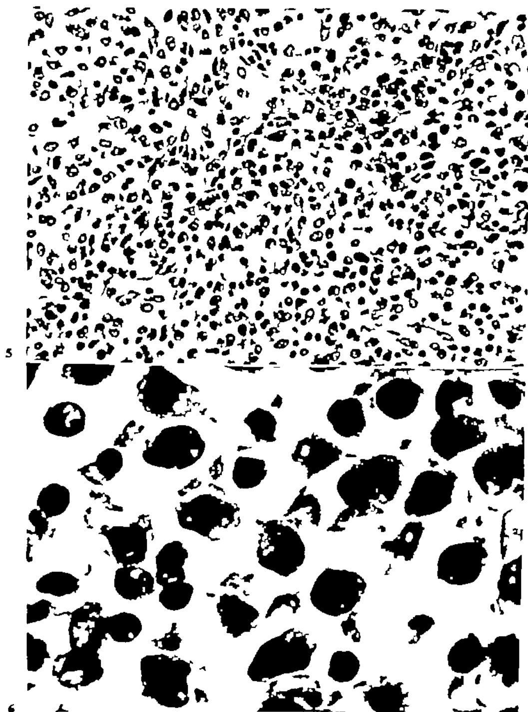


Fig. 5 Autopsy specimen of an abdominal lymph node HE. × 100

Fig. 6. Higher magnification of the same specimen as in figure 5 HE. × 400.

6) Mitotic figures were seen rarely as well as marked atypias (single trinucleated cells). In addition to such cells, there were lymphocytes most of which exhibited the appearance of centroblasts and centrocytes. Immunofluorescence studies performed on tissue materials obtained at autopsy showed strong IgM/λ positivity only in the lymph node specimens. The search for amyloid (both of the apud and immuno type) gave negative results in all the examined tissues.

Discussion

Nodular lymphoma appears now to be clearly established as a B cell neoplasm originating from follicular centre cells [13 14] The occurrence of a serum M component in nodular lymphomas seems to be a much less common finding as compared to diffuse lymphomas [1] There are however occasional reports of serum monoclonal immunoglobulins belonging to the IgG or IgM class in association with nodular lymphomas [11, 13, 16, 28]. In the case under discussion, although the immunoelectrophoretic analysis of the patient's serum had not been performed in the first admission, it is highly probable that the same M component identified later was present at the time of the initial diagnosis of nodular lymphoma. This conclusion seems to be justified by the abnormal scanning diagram of the serum protein separation obtained by a microzone electrophoretic technique, by the results of the quantitation of serum immunoglobulins, and by the positive cold agglutinin test.

The transformation of the histological picture, consisting most frequently in the development into an 'unfavorable' or 'high-grade' variety of lymphoma, has been reported to occur during the course of the disease in a significant proportion of patients with nodular lymphoma [5 7 19 25] Patients with malignant lymphoma have also been reported to occasionally exhibit the simultaneous occurrence of two distinctive cytological patterns in different sites, as well as in different parts of the same biopsy specimen [10 13] It is, however an unanswered question whether such lesions all derive from the same neoplastic clone or represent biologically distinctive neoplasms arising from separate clones.

The demonstration of the same monoclonal immunoglobulin in the cells of histologically dissimilar neoplasms occurring consecutively in the same patient [2, 3 28] may be taken as strong presumptive evidence of monoclonality thus supporting the hypothesis that the second cytological pattern is probably related to the original B cell proliferation and does not represent the emergence of a second malignant clone. It is well known, however, that the proof of monoclonality within a B cell population would require the demonstration that all the cells, regardless of their morphology exhibit an extremely restricted clonal marker such as the immunoglobulin idiotype. The results of combined morphological, immunological and immunohistological studies of malignant B cell neoplasms have provided evidence that a 'monoclonal' cell population does not necessarily have uniform appearance [26, 27] Thus, variations in morphological types may be related to changes in the relative proportions of the different forms of the B lymphocyte within the neoplastic clone

Although direct evidence is lacking, it is possible that the two different histopathological patterns observed in our patient may be biologically related. On the other hand, clear-cut boundaries between nodular (centroblastic/centrocytic) lymphoma and LP

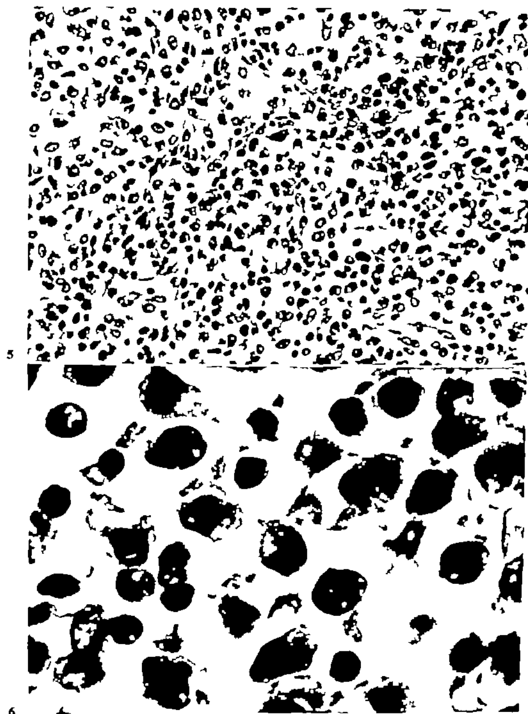


Fig. 5. Autopsy specimen of an abdominal lymph node. HE. × 100.

Fig. 6. Higher magnification of the same specimen as in figure 5. HE. × 400.

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immunocytoma may sometimes be difficult to draw in view of the recognized origin of nodular lymphoma from the follicular centre which is the site of production of the precursors of plasma cells. Morphological evidence has been provided of the occurrence of borderline cases between centroblastic/centrocytic lymphoma and LP immunocytoma [13].

Apart from any speculation about the possible relationship between the two histopathological patterns occurring consecutively, some unusual findings observed in our case seem to make it of interest when examined with respect to current concepts of B lymphocyte-derived neoplasms.

The quite rapid downhill course of the disease from the time of clinical presentation appears to be rather unusual for nodular lymphomas. No period of clinical remission intervened before the development of the second histological pattern. Postmortem examination revealed the persistence of the original histological pattern in the bone marrow, liver and spleen while the transformation occurred in the abdominal lymph nodes. The variation in the histological picture did not consist of transformation to a high-grade variety of lymphoma, as more frequently described in nodular lymphoma [5, 7, 19, 25]. The change, on the contrary, was characterized by the development of a functionally more differentiated B cell neoplasm.

Finally, a further interesting feature of the patient reported here was the biological activity of the serum monoclonal immunoglobulin which appears to have affected the course of the disease to a great extent.

The occurrence of monoclonal immunoglobulins exhibiting cold autoantibody activity directed against antigens of the erythrocyte membrane is a well recognized finding in some lymphoproliferative disorders. In these instances the monoclonal cold agglutinins belong mainly to the IgM class, usually with the *K* light chain, and most frequently possess specificity for the I antigen [17, 21, 24]. Monoclonal IgM cold agglutinins with the λ light chain type have also been reported to occur occasionally [4, 8, 12, 15, 18, 20, 24]. It is of interest, however, that IgM/λ cold agglutinins appear to exhibit some characteristics distinct from the more commonly encountered IgM/*K*. IgM/λ cold agglutinins, in fact, are very rarely directed against the I antigen [4, 8, 12, 15, 18, 24]; they are sometimes also cryoprecipitable [8, 12, 15, 18, 24] and have been frequently found in patients with malignant lymphomas with short survival time [18, 20].

In the case under discussion the serum IgM/λ M component can be regarded as an anti-non I cold agglutinin. The anti-Pr specificity may be strongly suspected since the patient's serum agglutinated adult and cord group O erythrocytes in equal strength, whereas the reactivity was abolished by protease and neuraminidase treatment of erythrocytes. Unfortunately, further studies by testing the agglutination activity with erythrocytes of animal species [21] were not performed in order to assess the precise specificity within the Pr system.

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Short Communications

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Systemic Lupus Erythematosus and Lymphoma

D W Milligan and J G Chang

Department of Clinical Haematology Manchester Royal Infirmary Manchester

Key Words. Lymphoma Systemic lupus erythematosus

Abstract. 2 cases of non-Hodgkin s lymphoma developing in young women with systemic lupus erythematosus (SLE) are described. The interval between the diagnosis of SLE and the development of lymphoma was 4 years and 4 months, respectively In 1 patient the lymphoma was localised primarily in the lung. It is suggested that the development of lymphadenopathy in a patient with SLE should be an indication for early lymph node biopsy

Introduction

Green et al. [1978] described 4 female patients and cited 8 others who had SLE and developed lymphomas in the course of the disease. Since then 3 further cases have been described in the Uk literature [*Barrett et al* 1978 *Gibbs and Seal*, 1978]. We present 2 further cases which have recently oc curred.

Case Reports

Case 1

A 15-year-old West Indian female born in the UK presented in 1974 with flitting arthritis and fever She later developed butterfly rash and alopecia. Investigations showed haemoglobin 10.3 g/dl, ESR (Westergren) 106 mm/h, ANF strongly positive, LE cells positive. A diagnosis of SLE was made and she responded well to prednisolone and remained well on a dose of 10 mg daily

In November 1978, painless lymphadenopathy developed in her neck, axillae and inguinal regions. Gland biopsy showed a diffuse lymphoblastic lymphoma and she was treated with combination of vincristine, adriamycin and prednisolone (VAP). The lymphadenopathy resolved and since March 1979 she has been in good health maintained on 6-mercaptopurine, methotrexate, cyclophosphamide and prednisolone.

Case 2

In October 1978 22-year-old female presented with pleurisy fever and jaundice. The spleen was enlarged 6 cm. She was pancytopenic although the marrow showed active haemopoiesis. Further investigations showed alkaline phosphatase 160 IU, AST 150 IU, serum bilirubin 80 mmol/l, ANF positive (IgG 14 IU) and chest -ray normal. Since she was extremely ill she was empirically treated with steroids.

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Dr M. Melato,
Istituto di Anatomia e Istologia Patologica,
Università di Trieste, Ospedale Maggiore,
Via Pietà 2.2,
I-34125 Trieste (Italy)

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The Reproducibility of two Methods of Studying Granulopoiesis Agar Culture and Labelling Index

D. Seigneurin and J. Léger

Laboratoire d'Hématologie, CHRU de Grenoble, Grenoble

Key Words. CFU-C · Myeloblast labelling index

Abstract. The overall reproducibility of two methods of studying granulopoiesis has been investigated, i.e. study of the granulocytic progenitor cells by the agar-culture technique and study of the myeloblast labelling index by autoradiography. The results obtained indicate that these two methods are reproducible for each step involved, from the time of marrow sampling right through to the final interpretation of the results.

Introduction

The long-term study of granulocytic progenitor cells or the myeloblast labelling index (LI) is of diagnostic importance or prognostic value in various diseases, and particularly in myeloid leukaemias, as stated by *Hartmann et al.* [3] and *Seigneurin and Hollard* [6]. However, in order to interpret the results obtained, it is essential that the overall reproducibility of these techniques be known for each step, from the time of marrow sampling up to the interpretation of the results.

The work reported here is a study of this reproducibility.

Materials and Methods

Materials

20 patients with acute myeloid leukaemia (type M1, M2, or M3 according to the FAB classification) were studied. All patients were studied twice at the time of diagnosis, before treatment, on day 0 and day 2. In 12 patients, the two techniques were carried out from the same marrow sample, i.e. sternal puncture (manubrium or first sternal piece) of total volume not exceeding 1 ml.

Methods

The technique of *Pike and Robinson* [4] was used to study the granulocytic CFU-C, i.e. double-layer agar culture, using Robinson's nutritive medium. Granulocytic colony-stimulating activity (CSA) was obtained from blood leucocytes of preselected voluntary donors. The final concentration of agar in the Feeder layer was 0.5%. The upper layer containing the target cells had final agar concentration of 0.3%. The same CSA was used for both cultures of each patient.

The marrow cells were suspended in heparinized Hanks' solution (phenol-free heparin; Roche, 20 U/ml). The cells were washed once with Hanks' solution and centrifuged at 1,500 rpm for 10 min. The pellet was resuspended in Hanks' solution to give minimum cellular suspension of 4×10^6 cells per millilitre.

All experiments were performed at two cellular concentrations, i.e. 2×10^5 and 5×10^4 per

The clinical improvement was dramatic and the laboratory tests reverted to normal. When the steroids were tailed off in December 1978, she relapsed with fever anaemia, pleurisy and nasal ulceration. Investigations showed haemoglobin 6.1 g/dl, white cells 1.8 × 10⁹/litre, platelets 25 × 10⁹/litre ANF positive (IgG 14 IU), DNA antibody 33 U/ml (normal < 10), chest x ray normal. Nasal mucosal biopsy revealed chronic inflammation. Prednisolone was re-started at 60 mg daily with clinical improvement and the blood count returned to normal.

In January 1979 she developed further malaise and intrapulmonary coin lesions were seen for the first time on the chest x-ray She then suffered a spontaneous pneumothorax. At thoracotomy a nodule was removed which was found to consist of diffuse histiocytic lymphoma. After a stormy post-operative period the patient was eventually treated with VAP but died 3 days later following a further pneumothorax. At necropsy the lungs were infiltrated by tumour as were the kidneys and adrenals. The lymph nodes, spleen and bone marrow were not involved. Histological interpretation was difficult but favoured extra-nodal diffuse histiocytic lymphoma.

Discussion

Since SLE is relatively common, it is curious that the association with lymphoma should be so uncommonly recognised. The reason for the association is obscure but it is possible that the abnormality of immune surveillance causing SLE may also predispose to the development of lymphoma. In case 2 the interval between the diagnosis of SLE and the development of lymphoma was short and it is possible that in this patient the 'SLE syndrome' resulted from the mechanism of tumour-derived antigenaemia, as suggested by *Green et al.* [1978] It is interesting that this patient developed primary malignant lymphoma of the lung. This condition is extremely uncommon [*Sakula*, 1979] but has been reported in one other patient with SLE [*Gibbs and Seal*, 1979] who also had undifferentiated non-Hodgkin s lymphoma.

In view of these cases, and despite the fact that benign lymphadenopathy frequently occurs in SLE [*Cammarata et al.*, 1963], we would suggest that the development of lymphadenopathy in a patient with SLE should be an indication for early lymph node biopsy

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Dr D W Milligan, BSC, MRCP
Department of Medicine, University of Leeds,
Martin Wing, General Infirmary
Leeds LS1 3EX (UK)

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D. Seigneurin and J. Léger

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Both colony (rare in this type of pathological study) and cluster formations were scored on day 7 Clusters were subdivided into microclusters (5–20 cells) and macroclusters (20–40 cells)

The ^{3}H thymidine LI was obtained using an autoradiographic technique [5] The marrow cells were incubated for 1 h at 37 °C with 10 μCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) After exposure for 8 days and Wright's staining, the percentage of leucoblasts containing more than three grains was determined per 1,000 leucoblasts.

The normal value for the LI of myeloblasts was 0.49 ± 0.04 which is consistent with those values found in the literature. The cultures and autoradiographs were evaluated by the same person. The statistical test used was Student's t test for paired comparisons.

Table I. Variations in the myeloblast LI and the number of CFU-C (clusters) in the marrow on day 0 and 2

Case No.	LI		CFU-C number	
	day 0	day 2	day 0	day 2
1	0.06	0.07	113	307
2	0 10	0.14	ND	ND
3	0 16	0.18	60 000	45 000
4	0 12	0.13	ND	ND
5	0.12	0.14	7	4 00
8	0.03	0 04	ND	ND
9	0.12	0 16	ND	ND
10	0 10	0.06	76	76
11	0.06	0.03	ND	ND
12	0.20	0.20	20 000	20 000
13	0.17	0.21	2 000	2 000
14	0.10	0.06	3	1
15	0.03	0.06	6 000	9 000
16	0.07	0.07	ND	ND
17	0.17	0.20	25	40
18	0.17	0.17	1 100	2 000
19	0.01	0.01	1	1
20	0.18	0.20	597	524

ND = Not determined.

Results

The myeloblast LI showed no significant variation. For a single patient, the largest variation was 0 04 The mean value for the LI was 0 099 on day 0 and 0 103 on day 2. The extreme values were 0 01 and 0.20 on day 0 and 0 01 and 0.21 on day 2.

The number of CFU-C (cluster number) on day 0 was no different from that on day 2 The extreme values were 1 and 60,000 clusters on day 0 and 1 and 45 000 clusters on day 2. Taking into consideration the difficulty in scoring and the culture method, it would appear that there is variation in the results obtained on day 0 and day 2 in only 1 patient (patient 5) This variation cannot be explained.

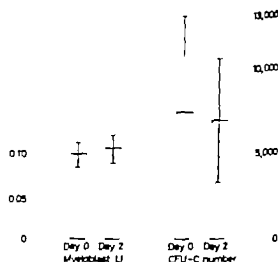


Fig. 1 Variations in the myeloblast LI and the number of CFU-C (clusters) in the marrow on day 0 and day 2 (mean $\pm$ SEM).

The size of the clusters and their cellular composition seemed constant from one sampling to the next.

Discussion

If one wishes to study the variations in a given parameter during the evolution of a disease, one must be certain that these variations are due to the disease and are not a result of the technique used. Furthermore, the methods used in this study involve many variable factors. The best possible standardisation of the techniques employed has been attempted. The results show that under these conditions, the study of granulocytic progenitor cells in agar culture and the measurement of the H-thymidine LI are satisfactorily reproducible. This holds true for the overall results of each of these methods, as well as for each step of each technique, from the time of marrow sampling to the interpretation of the final results.

Thus, it seems unnecessary to freeze each sample in order to test the series of samples for 1 patient at the same time as reported by *Kreutzmann and Fliedner* [2]. However it is absolutely necessary to use the same CSA throughout the long-term CFU-C study. It is indispensable to use a conditioned medium of good quality [1].

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D. Seigneurin, Laboratoire d'hématologie,
CHRU de Grenoble, BP 217 X,
F-38043 Grenoble Cédex (France)

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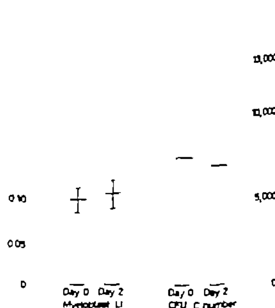


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D. Seigneurin, Laboratoire d'hématologie,
CHRU de Grenoble, BP 217 X,
F-38043 Grenoble Cédex (France)

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Sex and Splenectomy in Agnogenic Myeloid Metaplasia

J Benbassat and S Penchas

Department of Medicine A, Hadassah University Hospital, Jerusalem

Key Words. Actuarial survival Agnogenic myeloid metaplasia Splenectomy

Abstract. The available data of 338 cases of splenectomy in agnogenic myeloid metaplasia (AMM) published since 1940 were retreived from the literature and analysed. There were no marked differences between male and female patients as to their survival since splenectomy since the time of diagnosis or since the onset of the manifestations of AMM. Cardiac and thromboembolic complications caused death in 26% of the male patients, but only in 16% of the females, while hemorrhage and infections were more common causes of death among women. Leukemia developed in 11% of the males and in 6% of the female patients

Introduction

Silverstein et al [1] reported a longer survival after splenectomy of female patients with agnogenic myeloid metaplasia (AMM) Half of their 13 female patients survived at least 5 years after splenectomy while the average survival of 16 male patients was 2 years only Furthermore, the *mean duration of the illness before splenec*tomy was 4.5 years in women, but only 1 7 years in men. The authors were unable to identify any significant risk factors related to cardiac, renal, metabolic, hepatic or coagulation abnormalities that would make the male population of patients with AMM at a greater risk for splenectomy [1]

We have recently surveyed the literature on splenectomy in AMM and collected 338 cases (120 men 106 women and 112 patients in whom the sex was not reported) published since 1940 In the present report we compare male and female patients as to their survival, indications for splenectomy and causes of death

Methods

Reports of splenectomy in AMM published since January 1940 were retreived from the literature as detailed previously [2]. When available, clinical, laboratory and follow-up data were compiled for each individual published case according

to a predesignated format, and a digital computer was used for the analysis of the data. Actuarial survivorship curves since splenectomy since the time of diagnosis and since the onset of symptoms were constructed for cases with availabl data as described by *Berkson and Gage* [3].

Results

Breakdown of age, hemoglobin level, platelet number uric acid, splenic weight, vitamin B_{12}, serum alkaline phosphatase and albumin by sex revealed no significant differences between male and female patients. Portal hypertension was the indication for splenectomy in 7% of the males, but only in 1% of the female patients (table I). Cardiac and thromboembolic complications caused death in 26% of the male patients, but only in 16% of the females, while hemorrhage and infections were more common causes of death among women. Leukemia developed in 11% of the males and in 6% of the female patients (table II).

Table I. Indications for splenectomy in 166 published cases of AMM by sex

Indication for splenectomy	Males		Females	
	n	%		%
Mechanical discomfort	7	8	8	11
Thrombopenia	14	16	12	15
Anemia	43	49	43	55
Portal hypertension	6	7	1	1
Diagnostic	4	4	6	7
Splenic rupture	3	3	0	0
Routine splenectomy	11	13	8	11
Total	88	100	78	100

Table II. Splenectomy in AMM causes of death in 116 published cases by sex

Cause of death	Males		Females	
		%		%
Infection	24	37	24	47
Cardiac	8	12	4	8
Thromboembolic	9	14	4	8
Hemorrhage	8	12	9	17
Leukemia	7	11	3	6
Other tumor	3	5	3	6
Other causes	6	9	4	8
Total	65	100	51	100

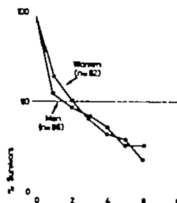


Fig. 1. Postoperative survival in published cases of splenectomy in AMM by sex.



Fig. 2. Survival since diagnosis in published cases of splenectomy in AMM by sex.

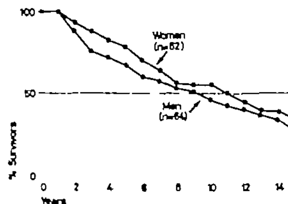


Fig. 3 Survival since the onset of manifestations in published cases of splenectomy in AMM by sex.

The median *postoperative* survival was 1 5 years in 96 males with available data and 2.0 years in 82 female patients (fig. 1) Survival *since diagnosis* was 5 1 years in 53 men and 5.8 years in 46 women (fig 2) Survival since *the onset of manifestations* was 9.2 years in 64 men and 11 0 years in 62 women (fig. 3) The *postoperative mortality* was 3 1 and 4 9% for male and female patients, respectively during the first 2 days after splenectomy The mortality within the first 3 months after splenectomy was 20.8 and 18.3% for male and female patients respectively

Discussion

Our results conform with previous observations that portal hypertension [4] and leukemia [1] are more common among male patients with AMM Female patients had a somewhat longer survival after splenectomy than males. However the differences were neither marked nor statistically significant. Furthermore, there were no differences in the postoperative mortality of male and female patients with AMM.

These findings are at variance with those of *Silverstein* and co-workers [1 5] who reported that survival after splenectomy was twice as long in female patients with AMM as in males. This discrepancy may be due to the relatively small sample (29 patients) on which the conclusions by *Silverstein and ReMine* [5] are based, and to the differences in age and indications for splenectomy between their male and female patients. The results of the present survey of a large number of published cases (338 patients) suggest that women with AMM do not appear to be better candidates for splenectomy than male patients with this disease.

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J Benbassat, Department of Medicine A, Hadassah University Hospital Jerusalem (Israel)

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Life-Threatening Hypophosphatemia in a Patient with Acute Myelogenous Leukemia

D Aderka, Y Shoenfeld, M Santo S Berliner M Shaklai, A Weinberger and J Pinkhas

Department of Internal Medicine 'D' Sackler School of Medicine, Tel Aviv University
Beilinson Medical Center Petah Tikva

Key Words. Hypophosphatemia Acute myelocytic leukemia

Abstract. A patient with acute myelogenous leukemia developed severe hypophosphatemia manifesting by extreme weakness, confusion, loss of sphincter control, nuchal rigidity hyperesthesia, hemolysis, congestive heart failure and liver dysfunction. The possible causes for this condition were starvation, parenteral glucose and saline administration, sepsis, hypokalemia and treatment with acetazolamide. A dramatic improvement was noted following phosphate administration.

The prolonged life expectancy of patients with acute leukemia permits the emergence of various electrolyte disturbances [1] We herewith report a patient with acute myelogenous leukemia in whom severe and life-threatening hypophosphatemia developed, followed by a dramatic improvement after phosphate administration.

Case Report

A 49-year-old woman suffering from acute myelogenous leukemia, with peripheral white blood count of 28,000 WBC/mm (97% myeloblasts) and 11.5 g/dl hemoglobin had on admission 4.5 mg/dl serum phosphorus and 9.1 mg/dl serum calcium. The latter levels remained practically unchanged following 5-day course of cytosine arabinoside 200 mg/day thioguanine 200 mg/day and daunorubicin 60 mg (administered on the 3rd day). A second course of the above-mentioned drugs was given 8 days later and was followed by the appearance of *Pseudomonas aeruginosa* septicemia. Antibiotic treatment consisting of gentamicin (240 mg/day), cephalotin (8 g/day) and carbenicillin (30 g/day) was started. Hypokalemia (2.1 mEq/liter) and oral candidiasis appeared; the patient was unable to swallow and therefore parenteral saline 0.9% 1,000 ml/day glucose 5%/1,500 ml/day with 6 g/day potassium supplements were administered. Uric acid crystals appeared in the urine and, in order to prevent kidney blockade by uric acid, intravenous acetazolamide 1.0 g/day was added. The patient's condition deteriorated gradually and 10 days later, marked weakness was noted: the patient was unable to perform the slightest movements. Several hours later incontinence, confusion and disorientation appeared, accompanied by hyperesthesia and nuchal rigidity The eyeground examination was normal. The pulse was bounding and regular 140 beats per minute, the cerebrospinal fluid was sterile, 4 lym-

phocytes per cubic millimeter were counted, and the protein and electrolyte levels were normal. The patient became jaundiced, her respiration was laborious and rales were heard over her both lungs. The chest x ray examination was consistent with congestive heart failure and revealed enlargement of the cardiac silouhette with engorgement of the pulmonary vessels. The blood gases were pO_2 80 mm Hg, pCO_2 20 mm Hg, pH 7.39 oxygen saturation 90%, bicarbonate 19 mEq/l. The routine laboratory examinations revealed hemoglobin 8.0 g/dl phosphorus 0.6 mg/dl, calcium 7.2 mg/dl, potassium 2.1 mEq/l, magnesium 2.19 mEq/l, total bilirubin 8.1 mg/dl (indirect 5 mg/dl) and SGOT 86 IU 24-hour urinary phosphorus excretion was 20 mg/l. The repeated blood cultures still grew *P aeruginosa*. 45 m*M* of phosphate salts were administered during the next 36 h followed by a dramatic improvement in the patient's condition. she regained full consciousness, the nuchal rigidity and the hyperesthesia disappeared, she regained sphincter control, the respiration became normal and the pulse stabilized at 90 beats/min. The phosphorus level rose to 1.9 mg/dl, the calcium was 7.6 mg/l, potassium 3.4 mEq/l, bilirubin 4.8 mg/dl, SGOT 61 IU and LDH 628 IU The patient's general condition gradually improved, the fever disappeared, the repeated blood cultures were sterile and 2 weeks later the phosphorus and calcium levels were normal (4.1 and 8.9 mg/dl, respectively).

Discussion

In this patient who had normal serum phosphorus levels on admission, severe hypophosphatemia with serum phosphate levels below 1 mg/dl developed after two courses of chemotherapy The spectrum of clinical manifestations caused by this condition included confusion, disorientation extreme weakness, hyperesthesia, congestive heart failure, hemolysis and liver dysfunction. It seems that the nuchal rigidity although previously unreported in patients with severe hypophosphatemia, was a manifestation related to this entity This assumption is based on the fact that there was no evidence of a leukemic meningeal infiltration and that the administration of the phosphate salts brought a prompt disappearance of the neurological findings. Hypophosphatemia, occasionally reported in patients with acute leukemia was attributed to the excessive uptake of phosphate by the leukemic blasts, with hyperphosphatemia, noted following initiation of chemotherapy and lysis of blasts [1]

None of the several conditions known to induce severe hypophosphatemia were presently observed, namely alcohol withdrawal, diabetes mellitus, pharmacologic PO_4 binding, diuretic phase after severe burns, hyperalimentation, nutritional recovery syndrome or severe respiratory alkalosis [2]. However several causes known to induce moderate hypophosphatemia were presently encountered, i.e hypocalcemia [1] starvation [2] glucose and saline administration [2, 3] sepsis [4] hypokalemia [5] and acetazolamide administration [6]

It seems that all of them played in concert to induce the severe hypophosphatemia in the presently reported patient.

Hypophosphatemia impairs the function of polymorphonuclear white blood cells leading to decreased resistance to infection [7 8] decreases the 2,3-dPG concentration in the red blood cells [9] impairs organ oxygenation [10] and causes severe abnormalities of platelet function [11] all of them contributing to the aggravation of the clinical condition of severely ill patients. This electrolyte imbalance could easily be corrected and was followed by an improvement in the patient's condition Multifactorial severe hypophosphatemia seems to be more frequent than recognized in the critically ill patients and has to be considered in similar cases.

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Prof. J Pinkhas, MD,
Department of Medicine 'D'
Beilinson Medical Center Petah Tikva (Israel)

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Original Papers

Acta haemat. 64 121 130 (1980)

Derangement of DNA Synthesis in Erythroleukaemia. Normal Deoxyuridine Suppression and Impaired Thymidine Incorporation in Bone Marrow Culture[1,2]

Kshitish C. Das Gurjeewan Garewal and Dipika Mohanty

Department of Haematology Postgraduate Institute of Medical Education and Research, Chandigarh

Key Words. Deoxyuridine suppression test DNA synthesis Erythroleukaemia Thymidine incorporation

Abstract. Deoxyuridine (dU) suppression test (i. e. ability of exogenous dU to suppress the incorporation of subsequently added ^{3}H thymidine into DNA) and the incorporation of ^{3}H-thymidine (^{3}H TdR) alone without dU were studied in bone marrow cultures from 10 patients with erythroleukaemia, 10 patients with vitamin B_{12}/folate-deficient megaloblastic anaemia and 10 haematologically normal subjects. Despite morphological resemblance between megaloblastosis in erythroleukaemia and nutritional megaloblastosis, the dU suppression values in erythroleukaemia were within normal range in contrast to abnormal dU suppression in vitamin B_{12}/folate-deficient megaloblastic bone marrows. The incorporation of ^{3}H-thymidine alone was significantly lower in erythroleukaemia than in normal or vitamin B_{12}/folate-deficient megaloblastic bone marrows. Autoradiographic studies showed that ^{3}H TdR labelling indices as well as mean grain count (MGC) of basophilic and polychromatic erythroblasts were significantly lower in erythroleukaemia than in normal or vitamin B_{12}/folate-deficient bone marrows. The reduced incorporation of ^{3}H TdR in erythroleukaemia erythroblasts was probably not due to deficiency of the salvage pathway enzyme, thymidine kinase, since MTX (10^{-6} *M*) which blocks the *de novo* pathway of thymine-DNA synthesis, enhanced the incorporation of ^{3}H TdR into erythroblasts in erythroleukaemia as well as in normal bone marrows. A high intracellular pool of thymidine-triphosphate (dTTP) due to defective DNA synthesis may allosterically inhibit thymidine kinase and ^{3}H TdR incorporation.

Introduction

Erythroleukaemia (DiGuglielmo's syndrome) is a myeloproliferative disorder characterized by a morphologically bizarre marrow erthythroid hyperplasia with ineffective erythropoiesis, and a variable involvement of myeloid precursors with a tendency to terminate in acute myeloblastic

[1] A preliminary report of part of this work appeared as an abstract in Proc. Annu. Meet. Indian Ass. Path. 1976, p. 12.

[2] This work was supported by research grant from the Indian Council of Medical Research.

Annoumcements

Announcement of the Gerhard-Domagk Prize

The Gerhard-Domagk Prize for clinical and experimental cancer research is herewith announced. The deadline for submitting contributions is December 31 1980

A pamphlet with the exact stipulations of the board of the Cancer Research Foundation Prof, Dr Gerhard Domagk, Münster can be obtained from. Priv Doz. Dr med. S. Ritter Medizinische Univ Klinik, Westring 3 4400 Münster (FRG)

The French Association of Hemophiliacs International Prize of FF 15,000 whose aim is to encourage medical research into the disease, will be awarded for the fourth time in 1981. The previous prizewinners have been.

1975 Dr Jean-Pierre Allain
La Queue-lès-Yvelines, France
1977 Dr Wagner
Chapel Hill, N.C., USA
1979 Drs. Blomm and Peake
Cardiff, England

The regulations, in French or in English, will be forwarded on request by the Selection Committee (Association Française des Hémophiles CNTS, 6, rue Alexandre-Cabanel, F–75739 Paris, Cédex 15 France).

The work submitted for the Prize must reach the Secretariat of the Selection Committee by March 15 1981, at the latest.

Original Papers

Acta haemat. 64: 121-130 (1980)

Derangement of DNA Synthesis in Erythroleukaemia. Normal Deoxyuridine Suppression and Impaired Thymidine Incorporation in Bone Marrow Culture[1,2]

Kshitish C. Das, Gurjeewan Garewal and Dipika Mohanty

Department of Haematology, Postgraduate Institute of Medical Education and Research, Chandigarh

Key Words. Deoxyuridine suppression test · DNA synthesis · Erythroleukaemia · Thymidine incorporation

Abstract. Deoxyuridine (dU) suppression test (i.e. ability of exogenous dU to suppress the incorporation of subsequently added ³H-thymidine into DNA) and the incorporation of ³H-thymidine (³H-TdR) alone without dU were studied in bone marrow cultures from 10 patients with erythroleukaemia, 10 patients with vitamin B_{12}/folate-deficient megaloblastic anaemia and 10 haematologically normal subjects. Despite morphological resemblance between megaloblastosis in erythroleukaemia and nutritional megaloblastosis, the dU suppression values in erythroleukaemia were within normal range in contrast to abnormal dU suppression in vitamin B_{12}/folate-deficient megaloblastic bone marrows. The incorporation of ³H-thymidine alone was significantly lower in erythroleukaemia than in normal or vitamin B_{12}/folate-deficient megaloblastic bone marrows. Autoradiographic studies showed that ³H-TdR labelling indices as well as mean grain count (MGC) of basophilic and polychromatic erythroblasts were significantly lower in erythroleukaemia than in normal or vitamin B_{12}/folate-deficient bone marrows. The reduced incorporation of ³H-TdR in erythroleukaemia erythroblasts was probably not due to deficiency of the salvage pathway enzyme, thymidine kinase, since MTX (10^{-} *M*) which blocks the *de novo* pathway of thymine-DNA synthesis, enhanced the incorporation of ³H-TdR into erythroblasts in erythroleukaemia as well as in normal bone marrows. A high intracellular pool of thymidine-triphosphate (dTTP) due to defective DNA synthesis may allosterically inhibit thymidine kinase and ³H-TdR incorporation.

Introduction

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leukaemia [4 29] Erythroid precursors show megaloblastoid changes unresponsive to therapy with vitamin B_{12} and folate A striking morphological similarity exists between megaloblastosis in erythroleukaemia, and megaloblastosis due to deficiency of vitamin B_{12} and folate (nutritional megaloblastosis) However cytogenetic, cytokinetic and enzymatic studies have suggested that megaloblasts in erythroleukaemia are of neoplastic origin [12, 18 19 24 26]

The molecular basis of megaloblastic erythropoiesis is still obscure It has been postulated that megaloblastosis is a morphological expression of deranged DNA synthesis from any cause [6 15] A qualitative abnormality of DNA synthesis has been shown to occur in short term cultures of bone marrow and PHA-stimulated lymphocytes from patients with nutritional megaloblastic anaemia due to deficiency of folate and vitamin B_{12} [8 10 21] This is characterized by impaired *de novo* synthesis of thymidine monophosphate (dTMP) from deoxyuridine (dU) as measured by the failure of exogenously added non-radioactive dU to suppress the incorporation via the salvage pathway of subsequently added ^{3}H thymidine (^{3}H TdR) into DNA (dU suppression test). This abnormality (abnormal dU suppression) is corrected *in vitro* by the addition of PteGlu, 5 methyltetrahydrofolate (5-methyl H_4PteGlu) and/or vitamin B_{12} depending on the nature of vitamin deficiency

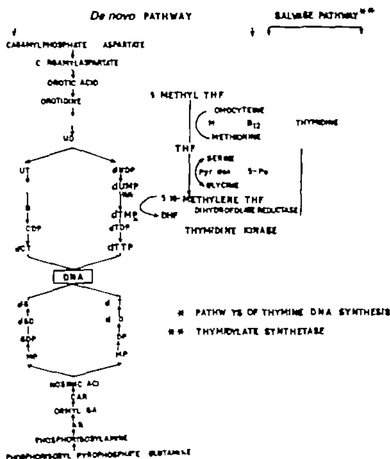


Fig. 1 Simplified diagrammatic representation of purine and pyrimidine biosynthesis. MP = Monophosphate DP = diphosphate TP = triphosphate d = deoxyribose A = adenine G = guanine C = cytosine T = Thymine U = uridine GAR = glycinamide ribonucleotide AICAR = aminoimidazol -4-carboxamide dUMP = deoxyuridine monophosphate dTMP = (deoxy) thymidine monophosphate dTTP = (deoxy) thymidine triphosphate.

Table I. Summary of haematological findings at the time of diagnosis of 10 patients with erythroleukaemia

Case No	Age years	Sex	Peripheral blood						Bone marrow		
			Hb g/dl	PCV (ratio)	retics %	WBC $\times 10^9$/l	myelo-blasts %	megalo-blast/ 100 WBC	M E ratio	myelo-blasts %	megalo-blastic changes
1	18	M	4 0	0 12	0 4	15	70	34	1.5 1	30	+ + + +
2	14	M	4 0	0 12	0 3	5	11	28	1 1 1	34	+ + + +
3	[illegible]	M	3.6	0.11	1 5	10	15	25	2 0 1	50	+ + +
4	28	M	5 0	0 15	0	2	9	38	0 6 1	20	+ + + +
5	37	F	6 0	0.18	0 6	5	30	19	1 8 1	37	+ + + +
6	24	F	7 4	0 23	2.5	25	37	26	1 2 1	41	+ + + +
7	39	M	6.8	0 20	1 7	31	52	20	0 9 1	35	+ + + +
8	45	M	8 6	0 6	3 1	43	58	28	1.3 1	57	+ + + +
9	32	F	9 1	0 28	1 4	29	32	50	1 4 1	37	+ + + +
10	29	M	8 7	0 27	0 9	18	12	31	1 1	40	+ + + +

The megaloblasts showed moderate to intense PAS positivity bi- and multinucleated megaloblasts were present in variable number in all bone marrows

causing megaloblastosis [7 21] The inter related biochemical pathways are shown in figure 1.

The present paper describes the results of dU suppression tests and *in vitro* H TdR incorporation studies in short-term cultures of bone marrows from patients with erythroleukaemia, who showed marked megaloblastoid' changes in marrow erythroblasts, morphologically indistinguishable from nutritional megaloblastosis.

Materials and Methods

Subjects Studied

Studies were carried out on 10 patients with erythroleukaemia before they were treated with any vitamins or antileukaemic agents. The results were compared with those of 10 haematologically normal subjects and 10 patients with nutritional megaloblastic anaemia due to deficiency of vitamin B_{12} or folate. Haematological findings in 10 patients with erythroleukaemia at the time of diagnosis are summarized in table I. The diagnosis was based on the following criteria: (i) anaemia, thrombocytopenia with or without leucocytosis or leucopenia; (ii) presence of megaloblasts and myeloblasts in the peripheral blood; (iii) bone marrow smears crowded with megaloblasts (> 30%), and myeloblasts with Auer bodies in some; (iv) normal serum and red cell folate, normal or high serum vitamin B_{12}.

Of the 10 patients with nutritional megaloblastic anaemia, 6 were folate deficient and 4 vitamin B_{12} deficient. They had moderate to severe anaemia (Hb level 6.8 ± 2.2 g/dl; PCV 21 ± 6.5%) with macro-ovalocytosis and neutrophil hypersegmentation in peripheral blood and florid megaloblastosis in the bone marrow

The 10 normal subjects were non-anaemic hospital patients who had normal levels of serum vitamin B_1 serum iron, TIBC, serum and red cell folat and serum protein. Bone marrow aspiration was done in all of them for cytogenetic studies as part of investigations for sterility and showed normocellularity with normoblastic erythropoiesis. Bone marrows were obtained from haematologically normal subjects t serve as controls for the biochemical studies carried out in the patients bone marrows. These 'normal' subjects came for investigations as to the cause of primary sterility Bone marrow aspirations were done in these pa-

leukaemia [4 29] Erythroid precursors show megaloblastoid changes unrespon sive to therapy with vitamin B_{12} and folate A striking morphological similarity exists between megaloblastosis in erythroleukae mia, and megaloblastosis due to deficiency of vitamin B_{12} and folate (nutritional mega loblastosis) However cytogenetic, cytoki netic and enzymatic studies have suggested that megaloblasts in erythroleukaemia are of neoplastic origin [12, 18 19 24 26]

The molecular basis of megaloblastic erythropoiesis is still obscure It has been pos tulated that megaloblastosis is a morpholog ical expression of deranged DNA synthesis from any cause [6 15] A qualitative abnor mality of DNA synthesis has been shown to occur in short term cultures of bone marrow and PHA-stimulated lymphocytes from patients with nutritional megaloblastic anaemia due to deficiency of folate and vitamin B_{12} [8 10 21] This is characterized by impaired *de novo* synthesis of thymidine-monophosphate (dTMP) from deoxyuridine (dU) as measured by the failure of exogenously added non radioactive dU to suppress the incorporation via the salvage pathway of subsequently added ^{3}H-thymidine (^{3}H TdR) into DNA (dU suppression test). This abnormality (abnormal dU suppression) is corrected *in vitro* by the addition of PteGlu, 5 methyltetrahydrofolate (5-methyl H_4PteGlu) and/or vitamin B_{12} depending on the nature of vitamin deficiency

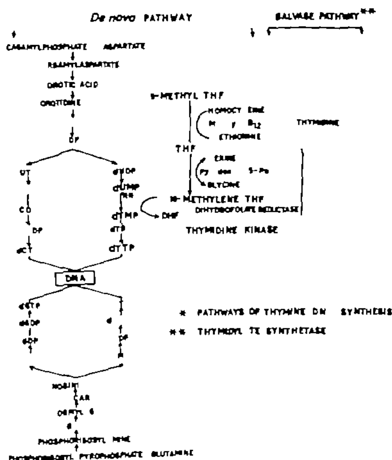


Fig. 1 Simplified diagrammatic representation of purine and pyrimidine biosynthesis. MP = Monophosphat DP = diphosphate TP = triphosphate d = deoxyribose A = adenine G = guanine C = cytosine T = Thymine U = uridine GAR = glycinamide ribonucleotide AICAR = aminoimidazole-4-carboxamide dUMP = deoxyuridi e monophosphate dTMP = (deoxy) thymidine monophosphate dTTP = (deoxy) thymidine triphosphate

resulted in partial correction, and 5-methyl-THF caused no correction, but combination of 5-methyl THF and cyanocobalamin caused almost complete correction. In the bone marrows cultures from patients with erythroleukaemia, dU caused almost complete suppression of ³H TdR incorporation into DNA, and the addition of PteGlu, 5-methyl-THF and cyanocobalamin had no further effect on the degree of dU suppression as in the normal marrows.

Incorporation of H TdR Alone and the Effect of MTX

The data is shown in table III. The incorporation of H TdR alone without addition of MTX was significantly lower in erythroleukaemia than in normal or vitamin B_{12} and folate-deficient megaloblastic bone marrows ($p < 0.01$). H TdR incorporation was, however much higher in vitamin B and folate deficiency than in normal bone marrows ($p < 0.01$). Preincubation of marrow cells with MTX (10^{-8} *M*) enhanced the incorporation of subsequently added H TdR into DNA in erythroleukaemia as well as normal bone marrows.

Autoradiographic studies of bone marrow also corroborated the above findings (table IV) H TdR labelling indices as well as MGC of basophilic and polychromatic erythroblasts were significantly lower in ery-

Table II. dU suppression test in bone marrow culture

Subjects	dU alone	dU + PteGlu	dU + vitamin B_{12}	dU + 5-methyl $H_4PteGlu^3$	dU + 5-methyl-H PteGlu + vitamin B_{12}
Normal (=10)	5.2 ± 1.7 (2.5–10.2)	4.9 ± 1.8 (2.2–9.7)	5.3 ± 2.1 (2.7–10.2)	4.5 ± 2.1 (2.1–10.0)	5.3 ± 1.9 (2.7–9.7)
Nutritional megaloblastosis folate-deficient (=6)	65.6 ± 21.6 (36.2–88.7)	7.2 ± 2.6 (3.6–11.7)	68.7 ± 25.3 (30.3–96.8)	6.7 ± 2.3 (4.1–12.0)	6.9 ± 2.4 (5.2–11.7)
Vitamin B_{12} deficient (=4)	72.4 ± 18.8 (30.5–90.2)	51.6 ± 12.8 (21.3–68.5)	39.8 ± 10.3 (18.5–57.2)	68.0 ± 22.3 (29.5–94.6)	6.5 ± 2.6 (3.4–10.8)
Erythroleukaemia (=10)	4.4 ± 2.2 (2.1–9.6)	4.8 ± 2.9 (2.4–10.0)	5.2 ± 1.9 (3.1–9.8)	4.8 ± 2.2 (2.4–9.5)	6.0 ± 3.1 (2.2–11.5)

³H TdR incorporation into DNA with dU and dU + folate/vitamin B_{12} are expressed as percentage of controls (³H-TdR incorporation without dU) Mean ± SD range is denoted in parentheses.

PteGlu = 50 µg/tube.

Cyanocobalamin = 1 µg/tube.

5-methyl-THF = 50 µg/tube (5-methyl-H_4PteGlu)

tients for chromosomal studies as a part of investigations for primary sterility No chromosomal abnormalities were found in them. These bone marrows were utilized immediately after aspiration for DNA synthesis and deoxyuridine suppression test and the data obtained (which was all normal) served as control.

Routine haematological studies were performed by standard techniques. Serum and red cell folate [23] and serum vitamin B_{12} were assayed microbiologically [1] Bone marrow aspirates were taken from the sternum and divided into aliquots for morphological and cytochemical studies, dU suppression test and ^{3}H TdR incorporation studies, and autoradiography

dU Suppression Test and ^{3}H TdR *Incorporation*

5 ml of the aspirated bone marrow was collected in cold heparinized 0.06 *M* Tris-buffered Hank's balanced salt solution (pH 7 4), and processed for measuring ^{3}H-TdR incorporation into DNA, and suppression test with dU These methods have been described in detail previously [7 8 21] All cultures were set up in triplicate. 3×10^6 cells were used in each culture tube, containing a final total volume of 1 ml. The cells were preincubated with 0.1 μmol of dU with and without PteGlu, 5-methyl-H PteGlu and vitamin B for 1 h, after which 1 μCi of ^{3}H TdR (specific activity 21.0 Ci/mmol Radiochemical Centre, Amersham, England) was added to each tube and further incubated at 37 C for 3 h. The control tubes were not preincubated with dU folate or vitamin B prior to the addition of ^{3}H TdR. The radioactivity of extracted DNA was measured in a Packard Tri-Carb Liquid Scintillation counter (Sr.No. C2425) and disintegrations per minut (dpm) were calculated from quench correction curve using an external standard. The incorporation of H TdR into DNA in cultures preincubated with dU with and without added folate or vitamin B_{12} was compared with and expressed as percentage of incorporation in the controls (i.e. replicate cultures without dU). DNA content was measured in an UV spectrophotometer at 268 nm using crystalline calf thymus DNA (Sigma Chemicals, USA) as standard.

The effect of methotrexate (MTX) on H TdR incorporation into DNA was studied by preincubating replicate cultures with and without MTX (10^- to 10^{-4} *M*) followed by addition of 1 μCi ^{3}H TdR (21 Ci/mmol) and incubating the cultures for a further 3 h. DNA extraction and measurement of radioactivity were done as described above [9 14 21].

Autoradiographic Studies

Approximately 0.05 ml marrow aspirate was mixed with an equal volume of Hank's balanced salt solution, pH 7 4 containing 200 U/ml each of penicillin and streptomycin and 5 μCi of ^{3}H-TdR (specific activity 5 Ci/mmol) In each case, replicate cultures were also set up to which both ^{3}H TdR and MTX (10^{-6} *M*) were added together These were incubated at 37 °C for 1 h, after which smears were prepared in glass slides with one frosted end, and fixed in methanol for 1 h. Autoradiography was carried out using Eastman Kodak NTB-3 dipping emulsion with an exposure time of 28 days at 4 °C. The slides were developed and fixed in Eastman Kodak developer and fixer then dried in air These were stained with May-Grünwald-Giemsa. The labelling index was determined by counting the number of labelled cells per 1,000 cells for basophilic and polychromatic erythroblasts, and the MGC was estimated from 50 labelled cells in red bone marrow

Results

dU Suppression Test

The results of dU suppression tests in bone marrow cultures of haematologically normal subjects, patients with nutritional megaloblastic anaemia due to deficiency of folate and vitamin B_{12}, and patients with erythroleukaemia are shown in table II In normal bone marrow dU (10^- μmol) suppressed H TdR incorporation into DNA almost completely (i.e approximately to less than 10% of controls) In nutritional megaloblastic marrows, dU suppression was markedly abnormal. This abnormality was almost completely corrected by *in vitro* addition of PteGlu and 5 methyl THF but not by vitamin B_{12} in folate deficiency in vitamin B -deficient marrows, the addition of PteGlu or vitamin B_{12} (cyanocobalamin)

resulted in partial correction, and 5-methyl-THF caused no correction, but combination of 5-methyl THF and cyanocobalamin caused almost complete correction. In the bone marrows cultures from patients with erythroleukaemia, dU caused almost complete suppression of ³H TdR incorporation into DNA, and the addition of PteGlu, 5-methyl-THF and cyanocobalamin had no further effect on the degree of dU suppression as in the normal marrows.

Incorporation of ³H TdR Alone and the Effect of MTX

The data is shown in table III. The incorporation of ³H TdR alone without addition of MTX was significantly lower in erythroleukaemia than in normal or vitamin B_{12} and folate-deficient megaloblastic bone marrows ($p < 0.01$). ³H TdR incorporation was, however, much higher in vitamin B_{12} and folate deficiency than in normal bone marrows ($p < 0.01$). Preincubation of marrow cells with MTX (10^{-8} *M*) enhanced the incorporation of subsequently added ³H TdR into DNA in erythroleukaemia as well as normal bone marrows.

Autoradiographic studies of bone marrow also corroborated the above findings (table IV). ³H TdR labelling indices as well as MGC of basophilic and polychromatic erythroblasts were significantly lower in ery-

Table II. dU suppression test in bone marrow cultures

Subjects	dU alone	dU + PteGlu	dU + vitamin B	dU + 5-methyl H_4PteGlu[a]	dU + 5-methyl-H_4PteGlu + vitamin B_{12}
Normal (n = 10)	5.2 ± 1.7 (2.5 – 10.2)	4.9 ± 1.8 (2.2 – 9.7)	5.3 ± 2.1 (2.7 – 10.2)	4.5 ± 2.1 (2.1 – 10.0)	5.3 ± 1.9 (2.7 – 9.7)
Nutritional megaloblastosis folate-deficient (n = 6)	65.6 ± 21.6 (36.2 – 88.7)	7.2 ± 2.6 (3.6 – 11.7)	68.7 ± 25.3 (30.3 – 96.8)	6.7 ± 2.3 (4.1 – 12.0)	6.9 ± 2.4 (5.2 – 11.7)
Vitamin B_{12} deficient (n = 4)	72.4 ± 18.8 (30.5 – 90.2)	51.6 ± 12.8 (21.3 – 68.5)	39.8 ± 10.3 (18.5 – 57.2)	68.0 ± 22.3 ([illegible]9.5 – 94.6)	6.5 ± 2.6 (3.4 – 10.8)
Erythroleukaemia (n = 10)	4.4 ± 2.2 (2.1 – 9.6)	4.8 ± 2.9 (2.4 – 10.0)	5.2 ± 1.9 (3.1 – 9.8)	4.8 ± 2.2 (2.4 – 9.5)	6.0 ± 3.1 (2.2 – 11.5)

³H TdR incorporation into DNA with dU and dU + folate/vitamin B_{12} are expressed as percentage of controls (i.e. ³H-TdR incorporation without dU). Mean ± SD; range is denoted in parentheses.

PteGlu = 50 μg/tube.

Cyanocobalamin = 1 μg/tube

5-methyl-THF = 50 μg/tube (5-methyl-H_4PteGlu)

Table III. Incorporation of ^{3}H TdR into DNA in cultures of bone marrow cells in the presence and absence of MTX

Nat re of lesion	^{3}H TdR incorporation into DNA, dpm		
	without MTX dpm/3×10^6 cells	with MTX (10^{-5} M) dpm/3×10^6 cells	percentage of control (i.e. replicate cultures without MTX)
1 Normal marrow (n=10)	28,520± 5 843 (20,550–40 850)	38,5.5± 8 175 (26,304–58 007)	135± 9 0 (126–152)
Erythroleukaemia (n=10)	20 824± 5 624 (14 956–28,054)	29 685± 6,987 (18,97 –40,638)	140±10.5 (120–161)
3 Nutritional megaloblastosis			
Folate deficient (n=6)	62,570±18 733 (38 462–88,553)	–	–
b Vitamin B$_1$ deficient (=4)	65,275±.2,551 (41,267–9..568)	–	–

Mean ± SD range is denoted in parentheses.

Table IV Results of autoradiographic studies on ^{3}H TdR incorporation without nd with added MTX (10$^-$ M) mean ± SD

	H TdR incorporation			
	Labelling index		mean grai count	
	without MTX	w th MTX	without MTX	with MTX
Basophilic erythroblasts				
1 Normal (=10)	66 7±4 1	68 8±5 4	30 9±5 9	49 5±7 4
Erythroleukaemia (=10)	45 8±9 7	50 1±8 4	17.5±4 3	6 5±5 5
3 Nutritional megaloblastosis				
Vitamin B deficient (n=4)	75 3±5 8	–	–	–
b Folat deficient (n=6)	77 5±6 8	–	–	–
Polychromatic erythroblasts				
1 Normal (=10)	38 8±6 9	40 5±6 6	16 ±3 5	.5 5±6
Erythroleukaemia (n=10)	13 5±5 4	15 7±6.5	9 ±..3	15 3± 7
3 Nutritional megaloblastosis				
a) Vitamin B$_{12}$ deficient (n=4)	33 5±8.9	–	–	–
b Folate deficient (n=6)	3 7±5 0	–	–	–

throleukaemia than in normal or vitamin B_{12}/folate-deficient bone marrows ($p < 0.01$). Addition of MTX significantly increased then MGC of erythroblasts in erythroleukaemia as well as in normal bone marrows ($p < 0.01$) but did not appreciably affect the H-TdR labelling indices of these cells. Erythroblasts in vitamin B_{12} and folate-deficient marrows tended to have higher ³H TdR labelling indices than in normal marrows, but the difference was not statistically significant, the MGC of erythroblasts in these vitamin-deficient marrows was significantly higher than in normal marrows ($p < 0.01$).

Discussion

The current studies show that despite the morphological resemblance between megaloblastosis in erythroleukaemia and megaloblastosis in vitamin B_{12} and folate deficiency the pattern of DNA synthetic abnormality in bone marrow cells in these two disorders are strikingly different. The dU suppression test (i.e. ability of exogenous dU to suppress ³H TdR incorporation into DNA) which essentially measures the effective conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) by the folate-dependent enzyme thymidylate synthetase, is a sensitive biochemical index of nutritional megaloblastosis due to deficiency of folate and vitamin B_{12} [6, 7 10 13 21] In the present study the dU suppression test yielded abnormal values in all 10 patients with nutritional megaloblastic anaemia, and the abnormalities were corrected by the appropriate *in vitro* additions of PteGlu, 5-methyl-THF and vitamin B_{12} as expected on the basis of the nature vitamin deficiency causing megaloblastosis. In the 10 patients with erythroleukaemia, the dU suppression of H TdR incorporation into DNA by bone marrow cells was within normal limits. These findings indicated that the uptake of exogenous dU its phosphorylation to dUMP and subsequent conversion to dTMP by the enzyme thymidylate synthetase were efficient so as to reduce the incorporation of subsequently added H TdR into DNA via the salvage pathway Thus, the *de novo* pathway of thymidylate synthesis was probably unimpaired in bone marrow cells of patients with erythroleukaemia, and the defective DNA synthesis in these cells is unrelated to that caused by deficiency of folate and vitamin B_{12}.

Normal dU suppression values in erythroleukaemia bone marrows were very briefly reported by *Waxman and Herbert* [25] in 6 patients and by *Wickramasinghe and Saunders* [28] in 2 patients without providing any data on H TdR incorporation by the marrow cells. The present data further showed that in contrast to vitamin B_{12} and folate-deficient bone marrow in which ³H TdR incorporation into DNA of erythroblasts was higher than normal, the incorporation of this radionucleoside in bone marrow of patients with erythroleukaemia was significantly lower than in normal bone marrows. The significant reduction of both labelling indices and MGC of erythroblasts in erythroleukaemia (table IV) suggest that mean H TdR incorporation into DNA per DNA-synthesizing erythroblast (S-phase cell) would be lower in erythroleukaemia than in normal or vitamin B_{12}/folate-deficient megaloblastic bone marrows. The results are consistent with those of previous investigators who reported a marked decrease in the fraction of DNA synthesizing cells among basophilic and early polychromatic erythroblasts in erythro-

T ble III. Incorporation of ^{3}H TdR into DNA in cultures of bone marrow cells in the presence and absence of MTX

Nature of lesions	^{3}H TdR incorporation into DNA, dpm		
	without MTX dpm/3×10^6 cells	with MTX (10^{-5} M) dpm/3×10^6 cells	percentage of control (i.e. replicate cultures without MTX)
1 Normal marrow (n=10)	28,520± 5 843 (20,550–40,850)	38,525± 8 175 (6,304–58,007)	135± 9 0 (126–152)
Erythroleukaemia (n=10)	20 824± 5 624 (14 956–28 054)	9 685± 6 987 (18 97 –40 638)	140±10.5 (1?0–161)
3 Nutritional megaloblastosis			
Folate deficient (n=6)	62,570±18 733 (38 462–88,553)	–	–
b Vitamin B deficient (=4)	65,275±22,551 (41,267–92,568)	–	–

Mean ± SD range is denoted in parentheses.

T ble IV Results of autoradiographic studies on H TdR incorporation without and with added MTX (10^{-} M) mean ± SD

	H TdR incorporation			
	Labelling index		mean grain count	
	without MTX	with MTX	without MTX	with MTX
Basophilic erythroblasts				
1 Normal (=10)	66 7±4 1	68.8±5 4	30 9±5 9	49 5±7 4
2 Erythroleukaemia (n=10)	45.8±9 7	50 1±8.4	17.5±4 3	6 5±5 5
3 Nutritional megaloblastosis				
Vitamin B deficient (=4)	75.3±5 8	–	–	–
b Folate deficient (=6)	77 5±6 8	–	–	–
Polychromatic erythroblasts				
1 Normal (n=10)	38 8±6 9	40 5±6 6	16 ±3 5	5 8±6
Erythroleukaemia (n=10)	13.5±5 4	15 7±6.5	9.. ± 3	15 3± 7
3 Nutritional megaloblastosis				
a) Vitamin B$_{12}$ deficient (n=4)	33.5±8 9	–	–	–
b Folate deficient (n=6)	32 7±5 0	–	–	–

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leukaemia bone marrow [11 22 24 26] This defect appeared to be more pronounced in the erythroblasts with megaloblastoid appearance a large fraction of which constituted a non proliferating cell population [22] Combined cytophotometric and autoradiographic studies further showed that this abnormality was mainly due to an abnormal accumulation of cells in G0 or G1 phase and the presence of U' cells, which were unlabelled with ^{3}H TdR had DNA content between diploid (2c) to tetraploid (4c) values [3 22, 24 26] The cytokinetic abnormalities of erythroblasts particularly megaloblastoid cells simulated those reported in leukaemic myeloblasts [2, 20] These and other lines of investigations suggested that the abnormal growth and proliferation of erythroblasts in erythroleukaemia probably resulted from neoplastic transformation of a stem cell common to both erythroid and granulocytic series [5 12, 18 19] It is possible that the impaired DNA metabolism in erythroid precursor cells specially megaloblastoid' erythroblasts in erythroleukaemia is a characteristic feature, fundamental to the neoplastic or leukaemic nature of these cells

The biochemical mechanism of deranged DNA synthesis in erythroleukaemia is still uncertain A balanced synthesis of purine and pyrimidine nucleotides is an important requirement for DNA synthesis in proliferating cells The nucleotide pools in the cells are maintained by *de novo* synthesis, incorporation of exogenous substrates by salvage pathways, and interconversions of different nucleotides (fig. 1) A complex pattern of control loops operate in regulating these processes. dTMP which, after a sequence of phosphorylation forms dTTP and is utilized for DNA synthesis, is in turn produced either by *de novo* synthesis from carbamyl-asparate or by phosphorylation of exogenous thymidine by the salvage pathway The two alternative pathways of thymidylate synthesis, the *de novo* and the salvage pathways, are interrelated by a common end product, dTTP which exerts a regulatory influence on both pathways by a feedback inhibition [17] This also accounts for the reciprocity of the two pathways [9] In deficiency of vitamin B_{12} and folate or in the presence of folate antagonists such as MTX, the impairment of the *de novo* pathway leads to increased activity of the salvage enzyme thymidine kinase [16 22] which in turn promotes increased incorporation of exogenous thymidine into DNA In contrast, the megaloblastoid erythroblasts in erythroleukaemia marrows showed reduced incorporation of exogenous ^{3}H TdR as compared to normal as well as vitamin B_{12}/folate-deficient megaloblastic bone marrows. The explanation for this finding remains speculative It is unlikely that deficiency of the salvage enzyme thymidine kinase is responsible for this biochemical abnormality because preincubation of the marrow cells with MTX caused a significant increase in the incorporation of ^{3}H TdR by the erythroblasts in erythroleukaemia as in the normal bone marrows. It is conceivable that a derangement of DNA synthesis in erythroleukaemic (neoplastic) erythroblasts may lead to a high intracellular concentration of dTTP which in turn would inhibit thymidine incorporation into DNA by a feedback process depressing the activity of thymidine kinase allosterically Studies on intracellular nucleoside triphosphate pools may reveal a clearer picture of the biochemical defect leading to the derangement of DNA synthesis in erythroleukaemia.

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leukaemia bone marrow [11 22, 24 26] This defect appeared to be more pronounced in the erythroblasts with megaloblastoid appearance, a large fraction of which constituted a non proliferating cell population [22] Combined cytophotometric and autoradiographic studies further showed that this abnormality was mainly due to an abnormal accumulation of cells in G0 or G1 phase, and the presence of U' cells, which were unlabelled with ³H TdR had DNA content between diploid (2c) to tetraploid (4c) values [3 22, 24 26] The cytokinetic abnormalities of erythroblasts particularly 'megaloblastoid cells simulated those reported in leukaemic myeloblasts [2 20] These and other lines of investigations suggested that the abnormal growth and proliferation of erythroblasts in erythroleukaemia probably resulted from neoplastic transformation of a stem cell common to both erythroid and granulocytic series [5 12, 18 19] It is possible that the impaired DNA metabolism in erythroid precursor cells specially megaloblastoid erythroblasts in erythroleukaemia is a characteristic feature fundamental to the neoplastic or leukaemic nature of these cells

The biochemical mechanism of deranged DNA synthesis in erythroleukaemia is still uncertain A balanced synthesis of purine and pyrimidine nucleotides is an important requirement for DNA synthesis in proliferating cells The nucleotide pools in the cells are maintained by *de novo* synthesis, incorporation of exogenous substrates by salvage pathways, and interconversions of different nucleotides (fig 1) A complex pattern of control loops operate in regulating these processes. dTMP which, after a sequence of phosphorylation forms dTTP and is utilized for DNA synthesis, is in turn produced either by *de novo* synthesis from carbamyl-asparate or by phosphorylation of exogenous thymidine by the salvage pathway The two alternative pathways of thymidylate synthesis, the *de novo* and the salvage pathways, are interrelated by a common end product, dTTP which exerts a regulatory influence on both pathways by a feedback inhibition [17] This also accounts for the reciprocity of the two pathways [9] In deficiency of vitamin B_{12} and folate or in the presence of folate antagonists such as MTX, the impairment of the *de novo* pathway leads to increased activity of the salvage enzyme thymidine kinase [16 22] which in turn promotes increased incorporation of exogenous thymidine into DNA In contrast, the megaloblastoid erythroblasts in erythroleukaemia marrows showed reduced incorporation of exogenous ³H TdR as compared to normal as well as vitamin B_{12}/folate-deficient megaloblastic bone marrows. The explanation for this finding remains speculative It is unlikely that deficiency of the salvage enzyme thymidine kinase is responsible for this biochemical abnormality because preincubation of the marrow cells with MTX caused a significant increase in the incorporation of ³H TdR by the erythroblasts in erythroleukaemia as in the normal bone marrows. It is conceivable that a derangement of DNA synthesis in erythroleukaemic (neoplastic) erythroblasts may lead to a high intracellular concentration of dTTP which in turn would inhibit thymidine incorporation into DNA by a feedback process depressing the activity of thymidine kinase allosterically Studies on intracellular nucleoside-triphosphate pools may reveal a clearer picture of the biochemical defect leading to the derangement of DNA synthesis in erythroleukaemia.

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Fanconi's Aplastic Anemia, Analysis of 18 Cases

S Gözdaşoğlu, A O Çavdar A. Arcasoy E. Babacan and O Sanal

Pediatric Hematology and Oncology Research Unit, Turkish Scientific and Technical Research Institute, Medical School of Ankara University Ankara

Key Words. Fanconi's aplastic anemia

Abstract. A total of 18 patients within the age range of 5–13 years, 12 male and 6 female, are diagnosed as having Fanconi's aplastic anemia on the basis of congenital abnormalities, pancytopenia, bone marrow hypoplasia, and chromosomal and hematologic analysis. The hereditary and familial basis of Fanconi's aplastic anemia was apparent in this series. Common abnormalities were growth retardation, café au lait spots, hyper pigmentations, microcephaly phalange deformities, mental retardation, and hypogenitalism chromosome abnormalities were detected in the majority of our cases. Mast cells were observed in the bone marrow in most of the patients. 1 case developed acute myelomonocytic leukemia.

Introduction

Fanconi's aplastic anemia is a familial pancytopenia associated with bone marrow hypoplasia and congenital malformations, originally described as occurring in 3 brothers by Fanconi in 1927 [1]

Several congenital abnormalities may be present in this disorder such as skeletal abnormalities, hyperpigmentation, renal malformations, microcephaly hypogonadism, and mental and growth retardations [2, 12]

This hereditary disease usually becomes hematologically manifest in the first decade of life [9 11] Structural chromosome abnormalities first described by *Schroeder et al.* support the hereditary basis of the disease [12]

The purpose of this paper is to present 18 patients with Fanconi's aplastic anemia, as the largest series reported from Turkey [5 6, 8]

Materials and Methods

The cases of Fanconi' aplastic anemia were diagnosed between 1963 and 1978 in the Hematology Section of the Department of Pediatrics. They were 12 males and 6 females; their ages ranged from 5 to 13 years. The diagnosis of Fanconi' aplastic anemia was made on the basis of congenital abnormalities, pancytopenia, and bone marrow

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Dr Kshitish C. Das, Professor and Head, Department of Haematology Postgraduate Institute of Medical Education and Research, Chandigarh (India)

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Hemoglobin F level was increased in all patients with a range of 2.8–27%/ (mean 11.7%). Chromosomal analysis showed marked chromosomal breaks, fragments, endoreduplication and dicentric centromeres in 13 cases, although no significant chromosome change could be detected in 5 patients (fig. 2, 3).

1 of our patients developed AMML and died in the hospital whereas the sister of a boy with Fanconi's aplastic anemia in our series also developed acute leukemia in an-

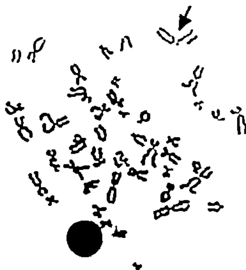


Fig. 2. Chromosomal structure abnormalities in child with Fanconi' aplastic anemia. Arrow indicates chromatid breaks and fragmentation.

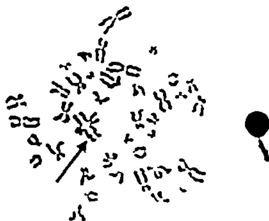


Fig. 3. Structural abnormalities in chromosome culture. Arrow indicates typical endoreduplication.

hypoplasia. Chromosomal analysis was performed in all cases. Hemoglobins F and A_2 were also measured in the majority of the cases by previously described methods [4]

Results

The congenital abnormalities observed in our patients can be seen in table I Consanguinity of the parents was observed in 9 patients and there was more than 1 case of aplastic anemia in 3 families. Figure 1 shows the pedigree of such a family Congenital malformations without anemia were also observed in two families.

Table I. Abnormalities in 18 cases of aplastic anemia

Abnormality	n
Growth retardation	18
Microcephaly	15
Café au lait spots	14
Mental retardation	9
Abnormalities of fingers (polydactyly syndactyly clinodactyly)	10
Deformities of the ear	8
Hypogenitalism	9
Malformations of toes	7
Microphtalmia	8
Hyperpigmentation	6
Renal anomalies	6
Thumb anomalies (absence or hypoplastic thumbs)	5
Vitiligo	3
Spina bifida	3
Hypoplastic spleen	2
Scoliosis	2
Strabismus	2
Sprengel's deformities	
Absent radius	1

Hematological findings are shown in table II All patients had pancytopenia with mean values of Hb of 4 7 g%, WBC 4 093/mm³ and platelet counts of 35,540/mm³ Bone marrow aspiration in 15 patients revealed marked hypoplasia while 3 had normocellular marrow It was interesting to note that 10 patients had a significant number of mast cells in the bone marrow megaloblastic changes in erythroid precursors were also observed in those patients who had erythroid foci in the marrow

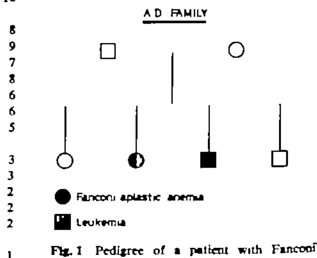


Fig. 1 Pedigree of a patient with Fanconi' aplastic anemia.

Table II. Hematological findings in Fanconi's aplastic anemias (mean values)

Hb g/dl	WBC/mm³	Pl telets/mm³	Serum iron µg/dl	UIBC µg/dl	Hb F %	Hb A
4 7	4 093	35.540	⁻79	92	11 4	‗7

Bone marrow Hypoplastic in 15 cases, normocellular in 3 cases, megaloblastic changes often observed in erythroid precursors with increased number of mast cells.

Hemoglobin F level was increased in all patients with a range of 2.8–27%/ (mean 11.7%). Chromosomal analysis showed marked chromosomal breaks, fragments, endoreduplication and dicentric centromeres in 13 cases, although no significant chromosome change could be detected in 5 patients (fig. 2, 3).

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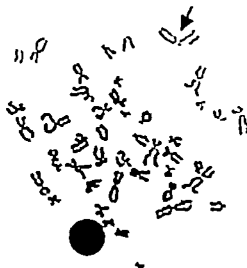


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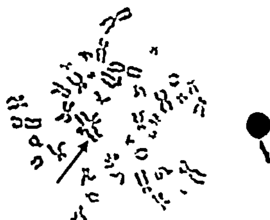


Fig. 3. Structural abnormalities in chromosome culture. Arrow indicates typical 'endoreduplication

other hospital (the type of leukemia was not clearly defined) All patients received combinations of corticosteroid and testosterone.

Discussion

The hereditary and familial basis of Fanconi s aplastic anemia was apparent in this series. In fact, consanguinity of the parents was noted in nine families and congenital anomalies without anemia were observed in two families It was interesting to note that more than one sibling was affected with Fanconi s aplastic anemia in three families. Autosomal recessive inheritance of the disease is generally accepted [11 12] Excess of males over females was noted in this series, a finding parallel to those reported in the literature [9]

The most common abnormalities observed in our cases were growth retardation, café au lait spots, hyperpigmentations, microcephaly deformities of phalanges including thumbs (fig. 4) renal malformations, mental retardation and hypogenitalism. Hypoplasia of the spleen interestingly was shown in 2 cases by scanning techniques

Structural chromosome abnormalities were detected in the majority of our cases (13 out of 18) (fig 2 3) The most common ones were chromosomal breaks, fragments, endoreduplication as have been described in the literature [8 12] However normal chromosome patterns were observed in one fourth of the patients.

The level of the fetal Hb was uniformly elevated in our cases with a mean value of 11.2% while Hb A_2 was normal (mean 2.7%) Although this is not a specific finding, it was our impression that Hb F was usually higher in this disorder than that ob-

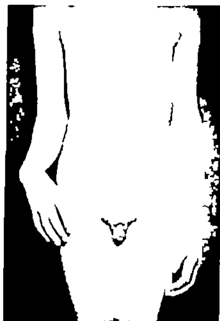


Fig. 4 Photograph of a 10-year-old boy with Fanconi s aplastic anemia showing the absences of radius and thumb on the right, hypoplastic thumb on the left hand and hypogenitalimus.

served in acquired aplastic anemias and/or in acute leukemias. Although all patients displayed pancytopenia in this series, it should be stessed that cytopenia involving one blood series may precede pancytopenia. Furthermore the patients with unexplained growth retardation with minimal malformations should be followed carefully as they may represent a incomplete expression of the disease In fact, one girl among our patients was followed at the outpatient department for 2 years for stunted growth with initially normal blood counts She eventually developed pancytopenia in the presence of bone marrow hypoplasia.

Growth hormone deficiency has occasionally been discovered in this disease [3]

The significant finding in the bone marrow of Fanconi's aplastic anemia was the presence of mast cells or tissue basophils which were not usually seen in other types



Fig. 5. 'Mast' cells in the bone marrow

of aplastic anemia (fig. 5). This finding could be used as a criterion for the diagnosis of a mild or incomplete form of the disease.

The development of acute leukemia in 1 of 18 cases of Fanconi's aplastic anemia is noteworthy and compatible with an increased risk of malignancy in this disease [1, 7 10] Interestingly the type of leukemia was acute myelomonocytic in our case, as reported previously [2]

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Doc. Dr Sevgi Gozdaşoğlu, Gaziosmanpaşa, Ilkadım, Sok. 9/10, Ankara (Turkey)

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Balanced Bone Marrow Globin Synthesis in Mideastern α-Thalassemia

R. Zaizov, M. Steinherz, B. Wollach and C. Kirschmann

Pediatric Hematology and Oncology Unit and Rogoff Wellcome Medical Research Institute, Beilinson Medical Center, Petah Tikva

Key Words. Globin synthesis · α-Thalassemia

Abstract. Hematological data and globin synthesis studies in 8 α-thalassemic children whose parents are from various regions in the Middle East are reported. All patients were devoid of Hb H. 5 of them had mild anemia, hypochromia and microcytosis and their blood α-/non-α-globin radioactivity ratios ranged between 0.56 and 0.75. The other 3 patients were hematologically normal with blood radioactivity ratios between 0.77 and 0.88. Bone marrow studies showed balanced globin chain synthesis for 6 of the patients and relatively more α-globin synthesis than in the blood for the remaining 2. The decrease in the relative synthesis of α-globin on erythroid cell maturation may relate to the molecular basis of α-thalassemia in the Middle East.

α-thalassemia is characterized by deficient production of α-globin in the hemoglobin synthesizing cells and its clinical manifestations seem to result from the intracellular accumulation of excess β-globin and from its eventual precipitation [1]. As the larger part of hemoglobin is synthesized within nucleated erythroid precursors, determination of globin synthesis in α-thalassemic bone marrow is expected to reveal the relationship between globin chain imbalance and the degree of the characteristic hematological abnormalities. Comparative studies of peripheral blood and bone marrow from thalassemic patients are also expected to reveal mechanisms that regulate hemoglobin biosynthesis during erythroid cell maturation.

Several investigators have examined globin chain synthesis in α-thalassemic erythroid precursors and have in most cases found it as unbalanced as in peripheral reticulocytes [2–4]. In the patients studied, thalassemia probably resulted from the deletion of α-globin genes and the consequent deficiency of specific mRNA in the immature hemoglobin synthesizing cells. In a previous study we have indicated that α-thalassemia in the affected Israeli Jewish communities differs by several features from that found in other populations [5] and *Orkin et al.* [6] have directly demon-

strated the presence of dysfunctional α-globin genes in the Mediterranean and Mideastern type [6]. The distinct molecular background may result in a different pattern of globin chain synthesis during erythroid maturation. We have indeed found in the bone marrow of 4 unrelated Israeli patients with Hb H disease significantly higher α-/non-α-globin radioactivity ratios than in their peripheral blood [7]. In this paper we report the radioactivity ratios in blood and bone marrow from 8 Mideastern carriers of α-thalassemia and show that bone marrow globin synthesis in 6 of them is balanced.

Methods

Bone marrow (1.2 ml) and blood (5 ml) were supplemented with glucose (0.5 mg/ml) and ^{14}C-leucine (354 mCi/mmol; 5 μCi/ml bone marrow, 1.5 μCi/ml blood) and incubated at 37 °C for 120 min. Cells were washed and preferentially lysed (4 vol of 1.5 mM $MgCl_2$, 90 sec) and their membranes removed (27,000 g, 30 min). Hemoglobin was precipitated by zinc acetate [8] and globin was isolated in acid acetone and chromatographed on CM-cellulose in urea [9]. Counting was performed in Insta-Gel (from Packard Instrument International) and the total radioactivity in each globin fraction was calculated.

Hematologic measurements were performed by standard methods as previously described [10].

Results

The children studied were 2–12 years old and have all had elevated concentrations of Hb Bart's at birth. At the time of this study no fast-moving hemoglobin could be detected in their blood and serum iron levels were within the normal range.

Table I shows the children's origin and some of their hematological data. Mild anemia, hypochromia and microcytosis are present in subjects 1–5 in whom Hb Bart's has been highest, whereas the other 3 subjects demonstrate no hematological abnormality.

Table II shows the α-non-α-globin radioactivity ratios for blood and bone mar

Table I. Origin and hematological data of α-thalassemia carriers

Subjects	Parent birthplace	Hb, g/dl	RBC, 10^{12}/l	MCH, pg	MCV, fl	Hb Bart at birth, %
1 O.A.	Iraq	10.5	4.36	24.1	78.0	5.2
2 K.C.	Yemen	11.5	4.86	23.6	74.0	5.5
3 B.C.	Yemen	10.2	5.30	19.2	73.4	5.5
4 I.C.	Yemen	10.6	5.38	19.7	66.9	5.4
5 R.E.	Iran/Afghanistan	10.0	5.48	18.2	63.9	3.0
6 L.O.	Yemen	11.7	4.15	28.2	93.9	1.4
7 A.H.	Romania/N. Africa	11.9	4.30	27.7	88.4	2.6
9 Y.V.	Egypt/Iraq	11.4	4.16	27.4	91.3	2.6
Nonthalassemic (25)[1]		12.89 ± 0.76[2]	4.13 ± 0.89	[illegible]9.88 ± 2.41	93.26 ± 8.79	<0.5

[1] Number of cases in parentheses.
[2] Mean value ± SE.

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The mechanisms that ensure balanced globin chain synthesis in α-thalassemic bone marrow may differ from those suggested to regulate globin synthesis in β-thalassemia. Excess β-globin in immature erythroblasts may be relatively stable and unlikely to undergo degradation [2, 3] Unstable α-globin mRNA could account for the radioactivity ratios in our patients bone marrow and peripheral blood and specifically for those determined in the subjects with normal red cell parameters and no anemia subjects 6–8. Indirect support for this assumption comes from the recent demonstration by *Orkin et al.* [6] of dysfunctional α-globin loci in Mediterranean and Mideastern α-thalassemic DNA, as transcription at such loci is likely to produce defective unstable α-globin mRNA. However the functioning of this or any other mechanism that may regulate globin synthesis in α-thalassemic bone marrow such as the suppression of β-globin synthesis or overproduction of α-globin by the nonthalassemic gene, calls for direct experimental evidence.

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Table II. Globin synthesis ratios in α thalassemic blood and bone marrow

Subjects	Peripheral blood α/non-α[1]	Bone marrow α/non-α
1 G A	0 68	1 00
2 K C	0.59	0 96
3 B C	0.56	0.77
4 I C	0 62	0 91
5 R.E	0 75	0.84
6 L.O	0 77	1 05
7 A.H.	0.88	0 94
8 Y Y	0 82	0 99
Nonthalassemic	1 07±0.05 (5)[2]	0 93±0 03 (4)

[1] Ratios of total radioactivity in globin fractions.
[2] Mean values ± SE number of cases in parentheses

row from these carriers of α thalassemia. The blood ratios range between 0.56 and 0 88 and are highest in subjects 6–8 whose red cell parameters are normal. Bone mar row radioactivity ratios for 6 of the carriers fall within the range determined for non thalassemic bone marrow while for the oth er 2 subjects B C. and R. E. they are low er 0 77 and 0 84 respectively Both these ratios, however are still significantly higher than the corresponding blood ratios.

Discussion

8 children with mild α-thalassemia syn thesized relatively more α-globin in their bone marrow erythroid precursors than in their peripheral reticulocytes 6 of them had bone marrow radioactivity ratios compara ble to those determined in nonthalassemic bone marrow indicating roughly balanced globin chain synthesis while the other 2, subjects B C. and R. E., had α-/non-α-globin radioactivity ratios of 0 77 and 0.84 respectively As these were determined in unfractionated bone marrow globin which is liable to contain radioactive nonheme prote in that chromatographs as β-globin [11] the possibility cannot be excluded that the bone marrow ratios for subjects B C. and R. E are erroneously low We however did not observe the presence of such nonheme pro-tein is bone marrow globin preparations from several other α-thalassemic patients and therefore consider the ratios listed in ta ble II to reflect a true variability in the ac tivity of bone marrow from these α-thalasse-mia carriers

A disparity in radioactivity ratios be-tween bone marrow and peripheral blood is known to occur in heterozygous β-thalasse-mia [12] and has been partly attributed to proteolysis of excess a-globin within early erythroid precursors [13 14] and to the op-eration within them of mechanisms that reg-ulate globin chain synthesis to reduce imbal-ance [15 16] Some type of regulation has also been suggested to occur in bone mar row cells of patients with Hb H disease, a severe form of α-thalassemia, to account for a discrepancy between the relative synthesis of α-globin in intact cells and in a cell-free system directed by RNA from them [4 7] On the other hand, contrary to the results reported in this paper 6 previously studied α-thalassemia heterozygotes had similarly low α-/non-α-globin radioactivity ratios in their bone marrow and blood [2–4] 5 of these patients were American blacks in whom a-thalassemia results from the dele tion of α-globin genes [17] and the 6th was a Caucasian whose origin has not been re-ported [3] The distinct findings in erythroid precursors from the α-thalassemia carriers studied by us probably relate to a different molecular background for their disease.

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An Estimation of β-Glucuronidase and N Acetyl β-D-Glucosaminidase Activity in Normal and Chronic Lymphocytic Leukaemia Lymphocytes

S I Dempsey A D Crockard and J M Bridges

Department of Haematology Royal Victoria Hospital, nd The Queen University Belfast

Key Words. Chronic lymphocytic leukaemia β-Glucuronidase Lymphocyte N-acetyl-β-D-glucosaminidase

Abstract. The lysosomal enzymes β-glucuronidase (EC 3.2.1.31) and N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) were estimated by biochemical and cytochemical means in the circulating lymphocytes of 20 control subjects, 19 cases of B cell and one case of T cell chronic lymphocytic leukaemia. Significantly lower levels of activity were observed both biochemically and cytochemically for each enzyme in B cell chronic lymphocytic leukaemia lymphocytes, whereas the values obtained for the T cell chronic lymphocytic leukaemic patient fell within the normal range. The absence of staining reaction for both enzymes in the majority of B cell chronic lymphocytic leukaemia lymphocytes contrasted with the uniform pattern of polar positivity obtained in T cell chronic lymphocytic leukaemia lymphocytes.

Introduction

The introduction of cell marker techniques has allowed the division of many lymphoproliferative disorders into B or T cell types. Investigation of the lysosomal enzymes acid phosphatase and β-glucuronidase in these conditions has given results which have been correlated with the presence of B or T cell markers. Cytochemical methods based on such findings can prove useful in the routine classification of lymphoid neoplasia and deserve further study

Thus, a cytochemical method [11] has been employed to estimate the β-glucuronidase content of normal and chronic lymphocytic leukaemia (CLL) lymphocytes and this technique has shown lower values in most cases of CLL [6, 20 21] A biochemical method to assess lymphocyte β-glucuronidase has similarly given low values in CLL [4 13] A further lysosomal enzyme N-acetyl-β-D-glucosaminidase (β-glucosaminidase) has been demonstrated both biochemically [1 5 14] and cytochemically [9 16] in normal lymphocytes. *Crockard et al.* [3]

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Dr. C. Kirschmann, Rogoff Wellcome Medical Research Institute, Beilinson Medical Center, Petah Tikva (Israel)

amined and graded as follows: grade 0: no activity; grade 1: 1 large or 1–5 small granules; grade 2: 5–10 small granules or 1 large plus 2–5 small granules; grade 3: 10–15 granules; grade 4: 15 or more granules.

The total activity score was the sum obtained by multiplying the number of cells by the observed grade [21].

Results

The immunological, cytochemical and biochemical findings are recorded in table I. Normal haematological and immunological results were noted for the 20 control subjects. A high percentage (> 60%) of SMIg positive cells were found in 19 of the 20 cases of CLL indicating B cell type. In 1 of the patients, 50% E rosetting cells were demonstrated indicating T cell disease.

Table I. Lymphocyte surface markers, β-glucosaminidase and β-glucuronidase cytochemical scores and biochemical activities (nmol 4-methylumbelliferone formed/10^8 cells/h; mean ± 1 SD)

	Control subjects	BCLL patients	TCLL patient
Sample number	20	19	1
Lymphocytes/µl	1,850	94,920	108,640
	± 650	± 86,950	
E rosettes, %	66	10	50
	± 9	± 8	
MRBC rosettes, %	2	43	4
	± 1	± 18	
Anti-Ig, %	17	67	3
	± 5	± 11	
β-Glucosaminidase			
Cytochemical score	139	29	182
	± 24	± 32	
Biochemical activity	21.9	4.6	14.6
	± 5.9	± 2.0	
β-Glucuronidase			
Cytochemical score	103	8	103
	± 22	± 9	
Biochemical activity	4.4	1.6	5.4
	± 1.5	± 0.5	

β-Glucosaminidase

The mean values for β-glucosaminidase activity assessed both biochemically and cytochemically in the CLL patients and controls are shown in table I. The enzyme activity measured biochemically was significantly lower in the B cell CLL cases ($p < 0.001$). Cytochemical scoring of the enzyme activity gave a mean for the controls of 139 (range 96–190), with 1 subject lying marginally above the range calculated for the mean ± 2 SD. The mean for the B cell CLL cases was 29 (range 1–128) and again 1 patient lay outside the calculated range for mean ± 2 SD. A significant difference ($p < 0.001$) was observed between the control and patient groups. The findings for the T cell CLL patient fell within the normal range using both techniques. The distribution of the cytochemical scores is illustrated in figure 1 and shows that approximately 70% of normal lymphocytes fell into grades 1 and 2, while approximately 80% of the lymphocytes in B cell CLL showed a complete absence of enzyme activity. The T cell CLL case had a notably even distribution of the enzyme.

β-Glucuronidase

The biochemical and cytochemical findings for β-glucuronidase are shown in table I. The activity of the enzyme measured biochemically was significantly lower ($p < 0.001$) in B cell CLL patients compared with normal donors. The cytochemical results showed a mean of 103 (range 60–148) for the controls with 1 subject

have shown significantly lower levels of this enzyme in the lymphocytes of B cell CLL compared with normal B lymphocytes. Although the cytochemical technique has been employed to investigate the lymphocyte β-glucosaminidase content in a number of conditions [7 10] no reference has been noted to its use in the study of CLL.

This study was therefore designed to investigate the cytochemical staining of β-glucosaminidase in CLL. The behaviour of β-glucuronidase was determined in parallel

Materials and Methods

20 patients with CLL were studied of whom 7 were on regular cytotoxic therapy at the time the samples were taken. 20 normal donors served as control subjects.

Lymphocyte Separations

Mononuclear cells were separated from 20-60 ml heparinised blood (10 U/ml) on Ficoll Isopaque as previously described [3]. Monocytes were removed from the mononuclear suspensions by the method of *Beutl et al.* [1]. Non-specific esterase using α-naphthyl acetate as substrate [22] and May-Grunwald-Giemsa staining indicated that monocyte contamination was always less than 3% in the final cell suspensions.

E Rosette Test

T lymphocytes were identified by their ability to bind sheep red blood cells (SRBC) sensitised with 0.14 *M* 2-aminoethylisothiouronium bromide (AET Sigma Chemical Company), using the method of *Pellegrino et al* [15]. The percentage of lymphocytes binding 3 or more SRBC was determined in a haemocytometer

Mouse Red Blood Cell (MRBC) Rosette

The method of *Shohat and Joshua* [17] was used and again the percentage of lymphocytes binding three or more MRBC was determined in a haemocytometer

Detection of Surface Membrane Immunoglobin (SMIg)

Lymphocytes were stained for SMIg using fluorescein-labelled sheep anti-human immunoglobulin serum (Wellcome Reagents Limited) as described earlier [3] Lymphocyte suspensions were observed under a Wild M20 fluorescent microscope and the percentage of immunofluorescing B lymphocytes determined.

N Acetyl-β-Glucosaminidase and β-Glucuronidase Assays

All lymphocyte suspensions were treated with 0.05% Triton X 100 and homogenised in a Potter Elvehjem homogeniser prior to assay N-acetyl β-glucosaminidase assays involved the incubation at 37 C for 10 min of 50 μl lymphocyte homogenate (1×10⁶ cells) with 100 μl 0.1 M citrate phosphate buffer pH 4.8 and 100 μl 1.25 mM 4-methylumbelliferyl 2-acetamide 2-deoxy-β-D-glucopyranoside (Koch Light Laboratories Ltd.). Reactions were terminated by the addition of 3 ml ice-cold 0.2 *M* glycine buffer pH 10 8. β-Glucuronidase was assayed similarly using 0 1 *M* acetate buffer pH 4.0 and 1.25 *M* 4-methylumbelliferyl-β-D-glucuronide (Koch Light Laboratories Ltd.). Fluorescence was measured using a Locarte Fluorimeter model 8/9 (Locarte Scientific Instruments) exciting at 360 nm and emitting at 450 nm.

Enzyme activity was expressed as nmol 4-methylumbelliferone formed/10⁸ cells/h.

N Acetyl-β-Glucosaminidase and β-Glucuronidase Cytochemistry

Fresh air-dried peripheral blood smears were fixed with cold formalin-methanol (7.3) at 4 C for 1 min, rinsed with distilled water dried at room temperature and kept at –20 C overnight. Blood films were thawed prior to incubation with the respective working solution. N-acetyl-β-glucosaminidase was demonstrated cytochemically using the method of *Reed and Bennett* [16] with naphthol AS-BI N-acetyl-β-glucosamide (Sigma Chemical Company) as substrate, coupled with fast garnet GBC. β-Glucuronidase was *demonstrated* using naphthol AS-BI-β D-glucuronide (Sigma Chemical Company) as substrate and hexazonium pararosanilin as coupler according to the method of *Yam and Mitus* [21]

All blood films were counterstained with 1 methyl green pH 4.2. 100 lymphocytes were ex

stained and graded as follows: grade 0: no activity; grade 1: 1 large or 1–5 small granules; grade 2: 5–10 small granules or 1 large plus 2–5 small granules; grade 3: 10–15 granules; grade 4: 15 or more granules.

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β-Glucosaminidase			
Cytochemical score	139 ± 24	29 ± 3[illegible]	102
Biochemical activity	21.9 ± 3.9	4.6 ± [illegible]0	14.6
β-Glucuronidase			
Cytochemical score	103 ± 22	8 ± 9	103
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in our 20 control subjects is somewhat lower than the figure of 147 recorded by *Flandrin and Daniel* [6] and 197 reported by *Yam and Mitus* [21] but in agreement with the figure of 95 noted by *Kohn et al.* [8] for subjects of a similar age. The significant reduction in cytochemical score for this enzyme in CLL lymphocytes has been reported by other authors [6, 11 21]

In scoring the cytochemical reaction for β-glucosaminidase in normal lymphocytes we were able to adopt the method advocated by *Yam and Mitus* [21] in their study of β-glucuronidase This method of scoring differs from that of *Lisiewicz* [9] who emphasised the presence of a diffuse pattern of positive staining in some lymphocytes and included this observation in his method. We found positivity was limited to a granular distribution by the technique of *Reed and Bennett* [16] The mean score for our 20 controls therefore cannot be directly compared with the results obtained by *Lisiewicz* [9] The cytochemical score for β-glucosaminidase showed a significant difference between the control and patient groups. This finding concurred with results obtained biochemically both in this and a previous study [3] Similar findings have been noted for other lysosomal enzymes, including acid phosphatase and α-naphthyl acetate esterase [4 19] In addition, reduced levels of adenosine deaminase have been reported in B cell CLL lymphocytes [12] in contrast to the increased levels of activity observed in lymphocytes from Waldenstrom's macroglobulinaemia patients [18] these differences may be a reflection of the varying stages of maturation attained by the B cell in the two distinct lymphoproliferative states.

The inclusion of a case of T cell CLL in our series allowed the study of β-glucosaminidase in this condition. T cell CLL has been described [2] as a chronic lymphoproliferative disorder differing in some clinical, cytological and immunological respects from the commoner B cell CLL. *Flandrin and Daniel* [6] recorded β-glucuronidase levels within the normal range in 2 out of a series of 20 cases of CLL. The lymphocytes were devoid of membrane-bound immunoglobulin suggesting possible T cell derivation and showed a uniform pattern of positive staining for the enzyme. In our case of T cell disease, β-glucuronidase and β-glucosaminidase showed a similar uniform pattern of staining with positivity occurring as a large granule at one pole of the cell. Biochemically both enzyme results fell within the limits of normal. The staining reactions of β-glucosaminidase, which have not been previously described in T cell CLL, would appear to conform to the pattern noted for β-glucuronidase and acid phosphatase [2] although this observation requires confirmation in a larger series of T cell CLL patients.

The use of cytochemical techniques to demonstrate the lysosomal enzymes acid phosphatase and β-glucuronidase appear to be of value in differentiating the common B lymphoproliferative disorders from the rarer T cell variety Our findings for β-glucosaminidase in CLL suggest that this enzyme parallels the behaviour of acid phosphatase and β-glucuronidase and could be employed in conjunction with these stains in the investigation of lymphoproliferative disease.

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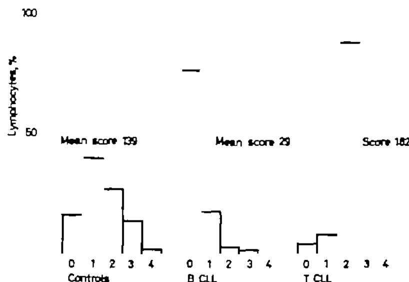


Fig. 1. Distribution of β-glucosaminidase activity acording to intensity of staining (grade 0–4).

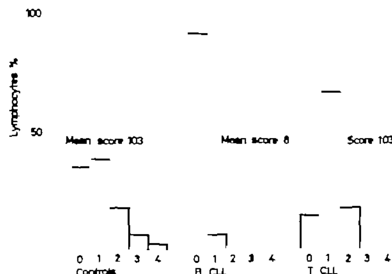


Fig. 2. Distribution of β-glucuronidase activity according to intensity of staining (grade 0–4).

lying marginally outside the calculated range using mean ± 2 SD and a mean of 8 (range 2–45) for the B cell CLL patients Again there was a significant difference ($p < 0.001$) Both biochemical and cytochemical results in the case of T cell CLL fell within our normal range Figure 2 shows the distribution of enzyme activity in the lymphocytes of the control and patient groups and reflects the pattern for β-glucosaminidase in normal and B cell CLL patients. The T cell CLL patient again showed a fairly even cellular distribution of the enzyme approximately 70% of the lymphocytes falling into grade 1

Discussion

Our findings of a reduced content of β-glucuronidase in CLL lymphocytes determined by biochemical means accord with those of *Douglas et al* [4] The mean cytochemical score of 103 for β-glucuronidase

in our 20 control subjects is somewhat lower than the figure of 147 recorded by *Flandrin and Daniel* [6] and 197 reported by *Yam and Mitus* [21] but in agreement with the figure of 95 noted by *Kohn et al* [8] for subjects of a similar age. The significant reduction in cytochemical score for this enzyme in CLL lymphocytes has been reported by other authors [6 11 21]

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Dr S I Dempsey Department of Haematology
Royal Victoria Hospital,
Belfast BT12 6BA (Northern Ireland)

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Dr S. L Dempsey Department of Haematology
Royal Victoria Hospital,
Belfast BT12 6BA (Northern Ireland)

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Autoradiographic Studies on Lymph Node Populations from Non-Hodgkin B Lymphomas[1]

Dennis Quaglino Andrea De Pasquale Lino Piccinini Massimo Tonelli and Marco Mugnaini

Istituto di Clinica Medica, University of Modena, Modena

Key Words. Autoradiography Cytokinetics Non Hodgkin B lymphomas

Abstract. The authors have investigated the cytokinetic behaviour of 23 cases of non-Hodgkin B cell lymphomas. In all cases diagnosis was established on the basis of histological cytoimmunological and cytochemical data. The study carried out with the aid of autoradiographic techniques, indicates that the various histological subclasses are characterized by a different metabolic and kinetic behaviour: the aggressiveness of the malignant lymphoma is closely related to the rate of growth of the lymphomatous cell populations, as assessed by the mitotic index and the labelling index with ^{3}H-thymidine. The study of the synthesis and metabolism of RNA provides not only useful information on the energy requirements necessary for the neoplastic growth, but, when employed in association with immunohistochemical techniques for the detection of intracytoplasmic Ig synthesis, it defines more accurately the functional characteristics and the differentiation potentialities of the neoplastic populations in different cases.

The prognosis of malignant lymphomas has hitherto relied almost exclusively on the histological characteristics of the malignant process and on the assessment of the clinical stage of the patient.

Quite recently with the purpose of obtaining objective information on those biological aspects of malignant cells which might have a direct bearing on prognosis and responsiveness to treatment, new lines of investigation have been pursued, such as the attempt by *Bloomfield et al* [1] to correlate the histological features of the tumour with the expression of the surface markers of neoplastic cells.

Similarly the observation that malignant lymphoproliferative disorders, even with a similar histological pattern show a different response to treatment and a highly variable clinical course, following an identical therapeutic regime prompted us to undertake an autoradiographic investigation on the kinetic and metabolic behaviour of lymph node populations from patients with different

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types of B lymphomas and we here report the preliminary results.

Material and Methods

The present study was carried out on 23 untreated cases of non-Hodgkin B lymphomas. In each case diagnosis was based on conventional histological and cytological examination, surface marker studies and cytochemical stains (non-specific esterases, acid phosphatases and PAS). Material from lymph node biopsies was processed as described in previous paper and used for autoradiographic studies, employing ^{3}H-thymidine and uridine 5-T [9].

The study of the percentage of cells labelled with ^{3}H-thymidine (LI), especially when combined

Table 1. Kinetic and metabolic findings in malignant cells from 23 cases of non Hodgkin B lymphomas (mean ± SD)

Histological diagnosis	Cell types	MI/ 10^3 cells	Autoradiographic findings			Cytoplasmic Ig content
			^{3}H TdR	uridine-5-^{3}H		
			LI/ 10^3 cells	mean nuclear grain count	percentage of N/C transfer	
ML lymphoplasmocytic (= 2)	LC	0	0	4.7 ± 0.8	0	− − −
	IB	19 ± 1	51 ± 5	65.7 ± 16.7	27.8	− − −
	PB	14 ± 2	33 ± 6	26.7 ± 2.4	24.2	+ − −
	PC	0	0	6.1 ± 1.8	16.4	+ + +
	LPC	0	0	5.6 ± 0.9	13.1	+ + −
ML immunocytic (= 1)	PB	11	28	31.3	27.1	+ + −
	PC	0	0	11	17.9	+ + +
ML lymphocytic (= 2)	LC, L		5 ± 3	29.1 ± 9.7	13.4	− − −
	LC, S	0	0	6.1 ± 4.9	0	(+) − −
ML centrocytic, diffuse						
Small cleaved cells (=)	CC S	0	4 ± 1	17.1 ± 4.1	6.1	− − −
Large cleaved cells (= 1)	CC L	29	48	35.3	11.3	− − −
ML centroblastic centrocytic, nodular (9)	LC	0	0	4.6 ± 1	0	− − −
	CC S	0	1.5 ± 1.4	12.1 ± 4.2	3.1	− − −
	CC L		22.2 ± 11.8	24.4 ± 9.5	12.6	− − −
	CB	27 ± 6	49.5 ± 5.7	36.8 ± 13.3	10.6	− − −
ML centroblastic (=)	CB	34 ± 3	53 ± 1	33.1 ± 8.6	11.5	− − −
ML immunoblastic (=)	IB	39 ± 9	31 ± 6	67.9 ± 3	4.4	− − −
ML Burkitt type (= 1)	BC	68	31	36.1	12.1	− − −

LC = lymphocytes IB = immunoblasts PB = plasmoblasts PC = plasma cells LPC = lymphoplasmocytic cells CC = centrocytes CB = centroblasts BC = Burkitt cells L = large S = small

Table II. Relationship between some clinical and kinetic aspects in 9 cases of nodular centroblastic/centrocytic malignant lymphomas

Name	Sex	Age	Clinical stage	^{3}H TdR LI/% of cells[1]				Therapeutic response[2]
				LC	CC, S	CC, L	CB	
S C	F	50	IIIA	0/16	0/70	4/9	46/5	good
Z T	M	48	IVB	0/21	1/68	18/9	45/7	poor
B.F	M	6	IIIA	0/36	1/59	39/4	51/1	poor
P R.	M	59	IVA	0/15	5/80	30 1	5 /4	poor
L.E.	M	56	IVA	0/37	1/58	19/4	40/1	good
R.Z.	M	63	IIIB	0/20	1/66	31/5	55/9	poor
B G	M	51	IIIA	0/48	2/47	16/1	46/4	good
C.S	F	70	IVB	0/26	1/69	9/4	58/1	good
M M.	F	42	IVA	0/38	2/52	34/3	53/7	poor
Mean value ± SD				0/28±11	1±1/63±10	±1 /4±3	49±6 4±3	

[1] Related to total malignant cell population.
[2] Following the same chemotherapeutic scheme

with the assessment of the mitotic index (MI), is capable of providing useful data on the proliferative activity of a neoplastic population.

The study of uridine incorporation and of the degree of nuclear-cytoplasmic (N-C) transfer of the label was carried out following the observation that in acute [10] and chronic myeloid [11] leukaemias the metabolic features are closely related either to the proliferative activity of the early components of the leukaemic cell population or to their residual capacity for differentiation. The uridine N-C ratio was obtained by dividing the mean cytoplasmic grain count × 100 by the mean total grain count. This value, on the basis of the results of our previous studies, was taken as an indication, at the cytological level, of the degree of ribosomal formation.

In the case of malignant lymphomas, uridine labelling may also reflect the synthesis of intracytoplasmic Ig. Therefore, a correct interpretation of the significance of uridine incorporation in lymphoma cells requires the parallel study of in-



Fig. 1. Malignant lymph node cells from a case of centroblastic lymphoma showing evidence of thymidine incorporation.



Fig. 2. Immunohistochemical demonstration of cytoplasmic Ig content in case f lymphoplasmocytic malignant lymphoma. Only 'mature' plasma cells are strongly positive.

tracytoplasmic Ig by the peroxidase-antiperoxidase technique described by *Sternberger et al.* [13]. I each case the LI was evaluated on 200 cells and the MI on 2,000 cells.

Results

Table I shows the various cell types present in the different histological varieties of non-Hodgkin B lymphomas, the MI the LI the degree of uridine incorporation as well as the percentage of N-C transfer of the la bel and finally the intensity of intracytoplasmic Ig peroxidase staining.

Table II relates the kinetic data to the different cell types present in 9 cases of centroblastic centrocytic lymphomas.

Discussion

Notwithstanding the considerable number of investigations which in recent years have been carried out in order to obtain more detailed information on the biology of malignant lymphoma cells, kinetic studies have been relati ely few [2–4 8, 9 12] Furthermore, many of the kinetic observa tions carried out in the past are no longer applicable to the morphological concepts which have stemmed from the Lukes and Collins and the Kiel classifications.

With regard to the present study it is necessary to distinguish between the kinetic behaviour of those cases of non-Hodgkin B lymphomas with a mixed cellular pattern and those with a predominantly uniform cell population.

In the former the monoclonal proliferation quite closely follows the differentiating pathway of the stimulated B lymphocyte [7] It is, however possible to observe a maturation arrest in some cell types, displaying an immunoblastic appearance, which are completely devoid of proliferative activ ity In malignant lymphoplasmocytic lymphoma there are also nuclear-cytoplasmic asynchronisms, since the synthesis and ex cretion of *Ig may be present in cells, defined* in table I as lymphoplasmocytic, which are morphologically similar to small lymphocytes that normally do not exhibit such an activity

With regard to those lymphomas with a predominant one cell population, immunocytomas and lymphocytic lymphomas are

almost inert from the kinetic point of view except for a small fraction, respectively of plasmoblasts and large nucleolated lymphocytes which acts as a self maintaining proliferating pool

The cells of immunocytomas as well as of lymphocytic lymphomas retain a certain degree of uridine incorporation which in the case of immunocytomas is probably related to their variable capacity to synthesize immunoglobulins.

Diffuse centrocytic lymphomas (small cleaved cells) are characterized by a low LI while mitoses are exceptional Probably these cells are mostly out of cycle or in a prolonged G_1 phase.

In diffuse centroblastic lymphomas (large non-cleaved cells), the LI appears to be very high, similar to the pattern which may be observed in normal centroblasts [7] while the MI lags behind. This discrepancy suggests either that these cells have an exceptionally long S period compared to the other phases of the cell cycle or that a considerable number of them undergo endoreduplication not followed by mitosis. In fact, the DNA content of part of these cells is frequently in excess of the tetraploid values, as shown by the cytofluorometric studies of *Riccardi et al* [12]

The centrocytes (small cleaved cells) and centroblasts (large non-cleaved cells) of centroblastic/centrocytic lymphomas show the same kinetic behaviour as in the unmixed histological varieties. This pattern does not differ significantly in the different cases. However in our present case material we have observed a variable fraction of large cleaved cells, the LI of which varies considerably in single cases (table II) Possibly these latter cells correspond to an intermediate stage in the transformation of centroblasts to centrocytes and therefore a high percentage of such cells or an increased proliferative activity on their part may indicate the existence of a more rapidly expanding process or a greater tendency to evolve towards a more aggressive form as a result of a loss of their differentiating capacity

In fact, the single case of centrocytic lymphoma with large cleaved cells (table I) showed kinetic data in keeping with a centroblastic lymphoma and a highly aggressive clinical course

The kinetic behaviour shown by centrocytes and centroblasts of centroblastic/centrocytic lymphomas seems to support the hypothesis of *Lennert* [5] according to whom centrocytes derive from centroblasts. Acceptance of the *Lukes and Collins* [6] concept on the derivation of centroblasts from 'stimulated centrocytes would imply that centroblasts, unable to undergo differentiation, but endowed with a high proliferative activity were capable of completely replacing the centrocytic component in a short time an event which does not usually take place.

Burkitt cells and lymphomatous immunoblasts have a high LI and proportionally an even higher MI The observation of labelled mitoses after only 1 h of in vitro culture, indicates an exceptionally short G_2 period.

Centrocytes, centroblasts and immunoblasts show a progressive increase in the rate of uridine incorporation and N-C transfer of the label, presumably in connection with the energy requirements linked to their proliferative activity however uridine incorporation in neoplastic immunoblasts may sometimes also be related to their variable degree of Ig synthesis [4] although the RNA turnover rate never reaches the intensity found in non-neoplastic immunoblasts.

The preliminary results obtained, espe-

cially in our group of centroblastic/centrocytic lymphomas, indicate the existence of different metabolic and kinetic patterns, even within cases showing the same histological features, which may variably influence the course of the disease and the therapeutic response.

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Dennis Quaglino, Istituto di Clinica Medica,
Università di Modena,
41100 Modena, Via del Pozzo 71 (Italy)

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Successful Pregnancy during Combination Chemotherapy for Hodgkin's Disease

Helene Daly Shaun R McCann T D Hanratty and Ian J Temperley

Federated Dublin Voluntary Hospitals and St. James' Hospital, Dublin

Key Words. Pregnancy Chemotherapy Hodgkin s disease

Abstract. Hodgkin s disease commonly presents in young adults and can be cured or brought into prolonged remission by radiotherapy chemotherapy or a combination of both. An increasing number of women are therefore liable to become pregnant during or following treatment for the disease. We describe a patient in whom pregnancy was successful in spite of treatment with chemotherapy during the latter half of the antenatal period.

Case Report

A 21 year-old patient was admitted to the Haematology Unit complaining of hoarseness and generalised pruritus for 3 months. Physical examination revealed a temperature of 38 °C, a diffuse erythematous rash on her arms and legs, gross bilateral cervical lymphadenopathy The liver was palpable 5 cm below the right costal margin and the spleen was palpable 6 cm below the left costal margin. Investigations revealed a haemoglobin of 10.0 g/dl with a normal white cell count and platelet count. The ESR was 85 mm/h. The alkaline phosphatase was 157 IU, the aspartate aminotransferase (AST) was 63 IU (normal 7-40) and the alanine aminotransferase (ALT) was 80 IU A chest X-ray showed marked mediastinal and paratracheal lymphadenopathy Lymph node biopsy revealed lymphocyte-depleted Hodgkin's disease. A bone marrow aspirate and bilateral posterior iliac crest marrow biopsies (Jamshidi needle) revealed normal marrow without evidence of Hodgkin's disease. A liver and spleen scan confirmed enlargement of both organs although no specific focal defects were detected.

Because of amenorrhoea a pregnancy test was done. This was positive and clinical evaluation confirmed a pregnancy of approximately 18 weeks. Because of the pregnancy pipedal lymphangiography and liver biopsy were not carried out. A clinical diagnosis of stage IV B Hodgkin's disease was made and she was commenced on combination chemotherapy with cyclophosphamide, vincristine, procarbazine and prednisone as outlined in table I.

Following two courses of chemotherapy physical examination was normal apart from some residual hoarseness. Her antenatal course was uncomplicated. Full dose chemotherapy was continued throughout pregnancy and during a 2-week interval from therapy she was delivered of a 2 kg female infant. The Farr score revealed a gestational age of 37 weeks. Physical examination of the infant at birth and 1 year later revealed no abnormality and chromosomal analysis was normal. 1 year following delivery the patient's disease relapsed and was refractory to chemotherapy

Table I

	Dose given	Route	Days	Total dose given before delivery mg
Cyclophosphamide	1,000 mg ($650 mg/m^2$)	i.	1 and 8	7,000
Vincristine	2.0 mg ($1.4 mg/m^2$)	i.v.	1 and 8	14
Procarbazine	150 mg ($100 mg/m^2$)	p.o.	1–14 (inclusive)	7,350
Prednisone	75 mg ($50 mg/m^2$)	p.o.	15–28 (inclusive)	3,150

Weight = 53 kg surface area = $1.5 m^2$

Discussion

Pregnancy of 18 weeks gestation and widespread Hodgkin's disease with an unfavourable histology were diagnosed simultaneously in our patient. Hodgkin's disease had previously been reported during pregnancy and *Barry et al.* [1962] showed that there were no adverse effects of the disease on the pregnancy or of the pregnancy on the disease. In 1968, *Nicholson* reviewed 185 pregnancies in which cytotoxic drugs were given. 27 patients had Hodgkin's disease, but in only 3 cases was a patient treated with more than two drugs and procarbazine was not used in any case. He concluded that cytotoxic therapy after the first trimester was associated with low birth weight only. *Johnson and Filshie* [1977] reported a case in which Hodgkin's disease was treated from the 28th week of pregnancy with combination chemotherapy (mustine, vinblastine, procarbazine and prednisolone) and resulted in the birth of a normal infant. *Bender and Young* [1978] also reviewed the outcome of pregnancy in mothers treated with combination chemotherapy. They state that alkylating agents and antimetabolites were equally likely to cause fetal abnormalities. They refer to 1 patient treated with procarbazine in the first trimester with a normal outcome to the pregnancy. However, they also point out that fetal abnormalities and abortion were more likely to occur when chemotherapy was given during the first trimester of pregnancy.

Schein and Winokur [1975] refer to isolated descriptions of normal deliveries following treatment with procarbazine and vinca alkaloids. They stress, however, that because of major deficiencies in case reporting it is not possible to estimate accurately the risk with specific therapeutic modalities. In our patient, Hodgkin's disease was diagnosed early in the second trimester and in spite of full dose combination chemotherapy the pregnancy terminated in the birth of a low weight infant who was otherwise normal. In the presence of widespread disease and with the administration of large doses of cytotoxic drugs from the 18th week, it was possible for pregnancy to proceed to term without evidence of fetal malformation in this patient. The risk of teratogenicity from

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Discussion

Pregnancy of 18 weeks gestation and widespread Hodgkin's disease with an unfavourable histology were diagnosed simultaneously in our patient. Hodgkin's disease had previously been reported during pregnancy and *Barry et al.* [1962] showed that there were no adverse effects of the disease on the pregnancy or of the pregnancy on the disease. In 1968, *Nicholson* reviewed 185 pregnancies in which cytotoxic drugs were given. 27 patients had Hodgkin's disease, but in only 3 cases was a patient treated with more than two drugs and procarbazine was not used in any case. He concluded that cytoxic therapy after the first trimester was associated with low birth weight only. *Johnson and Filshie* [1977] reported a case in which Hodgkin's disease was treated from the 28th week of pregnancy with combination chemotherapy (mustine, vinblastine, procarbazine and prednisolone) and resulted in the birth of a normal infant. *Bender and Young* [1978] also reviewed the outcome of pregnancy in mothers treated with combination chemotherapy. They state that alkylating agents and antimetabolites were equally likely to cause fetal abnormalities. They refer to 1 patient treated with procarbazine in the first trimester with a normal outcome to the pregnancy. However, they also point out that fetal abnormalities and abortion were more likely to occur when chemotherapy was given during the first trimester of pregnancy.

Schein and Winokur [1975] refer to isolated descriptions of normal deliveries following treatment with procarbazine and vinca alkaloids. They stress, however, that because of major deficiencies in case reporting it is not possible to estimate accurately the risk with specific therapeutic modalities. In our patient, Hodgkin's disease was diagnosed early in the second trimester and in spite of full dose combination chemotherapy, the pregnancy terminated in the birth of a low weight infant who was otherwise normal. In the presence of widespread disease and with the administration of large doses of cytotoxic drugs from the 18th week, it was possible for pregnancy to proceed to term without evidence of fetal malformation in this patient. The risk of teratogenicity from

chemotherapy is far from clear *Sieber and Adamson* [1975] collected 14 conceptuses with major malformations following treatment with anti-tumour agents. However with the exception of anti folates the teratogenicity of the other agents used is uncertain. We would agree with their advice that therapy with cytotoxic agents should be withheld, if possible, until the end of the first trimester of pregnancy There is increasing evidence [*Sieber and Adamson*, 1975] that the incidence of acute leukaemia in patients treated for Hodgkin's disease is probably related to the use of chemotherapy especially if this is combined with radiotherapy However since the latent period for radiotherapy or chemotherapy induced leukaemia is up to 15 and 14 years, respectively [*Schwartz and Upton* 1958 *Galton and Spiers* 1971] it is very important that all children born to mothers receiving chemotherapy should be followed into adult life. It is possible that these normal children may significantly increase the size of the pool of humans who are at risk of developing malignancy

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Dr Shaun R. McCann,
Department of Clinical Haematology
St. James Street, Dublin 8 (Ireland)

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Polymorph Leucocyte Function in Uraemia and Jaundice

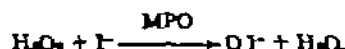
E. N. Wardle and Roger Williams

The Liver Unit, King's College Hospital, London

Key Words. Esterases · Jaundice · Leucocyte function · Myeloperoxidase · Uraemia

Abstract. Biochemical measures of leucocyte function have been examined using cells from patients with jaundice or uraemia in order to elucidate how toxins might affect those cells and vital enzymes such as NADPH oxidase, myeloperoxidase and esterases. Indices of function were depressed in fulminant hepatic failure, in acute uraemia and in some cases of alcoholic cirrhosis. Conversely there seemed to be an adaptive enzyme increase in chronic uraemia and obstructive jaundice. The toxins which affect leucocyte iodination and myeloperoxidase have been defined.

Patients with uraemia are known to be highly susceptible to infection [11] as are patients with some categories of jaundice, and the combination of jaundice with uraemia that is seen in patients with acute hepatic necrosis carries a great liability to pulmonary infection [5]. The capacity of the phagocyte to kill bacteria hinges on its ability to mount a respiratory burst of increased oxygen consumption and an increased utilisation of glucose by the pentose-phosphate shunt, and so to produce hydrogen peroxide and superoxide. In general these functions are related [4] and they correlate with the ability of the polymorph to incorporate inorganic iodide into organic form [15]. In a reaction that is catalysed by myeloperoxidase (MPO), which imparts the green colour to polymorphs, halide ions such as iodide are combined with oxygen and the hypohalite that is formed then iodinates the tyrosine residues of bacterial proteins.



$$H_2O_2 + I^- \xrightarrow{MPO} OI^- + H_2O.$$

Polymorphs that are deficient in NADPH oxidase (as in chronic granulomatous disease) or in MPO, neutrophiles that cannot produce hydrogen peroxide or those that have a defect in hexose monophosphate (HMP) shunt activity show defective iodination and bactericidal capacity [6]. Already one study in acute hepatic necrosis has shown defective HMP shunt activity [1]. In this study an assessment of simple tests for the study of leucocyte function and their

possible impairment in jaundice or uraemia has been made by examining glucose utilisation by the HMP shunt, the ability of cells to take up ^{14}C leucine [12] and their capacity for iodination [7] MPO and leucocyte esterase were also quantitated, and a study was also made of the effects of potential toxins on the former and on the related enzyme microperoxidase that is an integral part of cytochrome c.

Methods

Leucocytes were isolated from heparinised venous blood by sedimentation of the erythrocytes by addition of 3 / dextran and thereafter centrifugation of the plasma at 800 g for 5 min. Contaminating erythrocytes were removed by rapid hypotonic lysis using 5 ml cold 0.2%/ NaCl for 20 sec followed by the addition of 5 ml 1.6% NaCl. After repeating this procedure the cells were resuspended in appropriate buffer containing 5 m*M* dextrose. The neutrophil count was always in excess of 85%.

HMP Shunt Activity

The cells were suspended in Hanks' neutral salt solution containing 5 m*M* dextrose. HMP shunt activity was determined with and without stimulation by serum-treated zymosan using flasks containing 3–5 × 10^6 leucocytes in Hanks solution, 5.0 m*M* glucose and 0.05 μCi of glucose-1-^{14}C. Incubations were performed for 15 30, 45 min whilst the evolving $^{14}CO_2$ was adsorbed by an inner chamber containing 0.5 ml 1 *N* NaOH and 0.5 ml hyamine on a plug, and also for 30 min after termination of the reaction by addition of 60%/ citric acid. These adsorbent plugs were then dissolved in toluene scintillation medium prior to counting.

In additional experiments in which the effects of bilirubin, and of taurocholate and glycocholate on HMP shunt activity were examined, they were first added to the leucocytes in autologous serum 10 min prior to commencement of the assay by addition of glucose 1-^{14}C. Each was examined at 50 and 25 μM concentration.

Leucine Uptake

100 μl of leucocyte suspension (approx. 5 × 10^3 cells) was incubated with 100 μl Hanks' solution, to which at time zero was added 50 μl phosphate-buffered saline containing 0.1 μCi ^{14}C-leucine. The reaction was set up in a series of tubes and was stopped at 5 10, 15 min by the addition of 100 μl cold 10% trichloracetic acid (TCA) The precipitated proteins were then washed and resuspended thrice in cold normal saline containing 1.0 m*M* unlabelled leucine. Finally each cell button was dissolved in 0.1 *M* NaOH prior to counting in toluene scintillation fluid.

Leucocyte Iodination Capacity

Cells were resuspended in Krebs-Ringer phosphate buffer containing 5 m*M* dextrose and 8 × 10^{-8} *M* sodium iodide. To 0.2 ml cell suspension (approx. 5 × 10^3 cells) was added 0.1 ml pooled AB serum and to 'resting tubes' 50 μl plain buffer and to 'stimulated tubes 50 μl opsonised zymosan. After 15 min preincubation in a shaking water bath at 37 °C, 50 μl of ^{125}I (thiosulphate-free) was added and the tubes were incubated for 30 min. The reaction was terminated by the addition of 0.1 ml of 0.01 *M* sodium thiosulphate and then 0.2 ml 5%/ TCA. The tubes were centrifuged to yield the protein deposit, which was then washed thrice by thorough mixing and resuspension in saline. The radioactivity of 'resting' and stimulated' tubes was counted in a gammacounter The former count was subtracted from the latter to give the 'stimulated iodination capacity' [7].

Cells from patients with uraemia or jaundice were studied both in AB normal serum and in their own sera. The effects of the differing sera on cells from normal persons and on those from other clinical entities were also studied. In studies designed to assess the effects of possible toxins and drugs on the leucocyte iodination capacity of normal cells doubling dilutions were made down to infinitesimally small concentrations in a volume of 0.2 ml, and the above volumes of cells and reagents were added for the assay The effect of each toxin was examined thrice in order to determin the concentration that would reduce iodination by 50%.

MPO Activity

Leucocytes from patients with uraemia or jaundice were counted and were then frozen and

thawed out thrice. After centrifugation the clear supernatant was used for MPO and esterase assays. MPO was assayed using guaiacol and o-dianisidine as substrates. 2 ml phosphate-buffered saline, pH 7.4, was placed in cuvettes at 37 °C in dual beam recording spectrophotometer and to the test cuvette was added 0.1 ml leucocyte supernatant, 0.5 ml guaiacol (20 mM) as hydrogen donor and finally 0.1 ml hydrogen peroxide to achieve final concentration of 0.6 mM and from that moment the increase in orange colour was followed at 470 nm for 10 min. The initial reaction rate and the V_{max} were recorded and the activity was expressed as micromoles of tetraguaiacol/min/10^6 leucocytes using an extinction coefficient of 25.5 $cm^2/\mu mol$ [9].

The effect of potential toxins on MPO activity was studied in the same system by adding them in 0.5 ml saline prior to the reaction. The aim was to record the concentration of each that would reduce MPO activity by 50%. Likewise, the effects of the toxins on microperoxidase (Sigma) were examined by using as hydrogen donor 0.3 ml of 0.3 m*M* o-dianisidine made up in methanol and then diluted with 2 ml phosphate buffer.

Esterase Activity

The leucocyte supernatant (0.2 ml) was incubated for 6 min at 37 °C with 2 ml 0.1 *M* phosphate buffer pH 6.5 and as substrate 1.0 ml β-naphthylacetate (2 mg in 20 ml). The reaction was terminated by the addition of 0.5 ml 1.0 *M* TCA and the naphthol in the supernatant was read at 312 nm in comparison with an unincubated blank.

NADPH Oxidase Activity

0.2 ml leucocyte supernatant was added to 33 mM phosphate buffer pH 5.5 [2], and the decrease of absorbance at 340 nm was followed after the addition of NADPH to achieve final concentration of 0.1 *M*. The effects of bilirubin, taurocholate and glycocholate were examined at 2.5, 5.0 and 10 μ*M* concentrations.

Results

HMP Shunt Glucose Oxidation

Table I presents the results of the study of pentose-phosphate shunt activity in the

Table I. HMP shunt oxidation of glucose-1-^{14}C with opsonised zymosan stimulation

		nmol/h/5 × 10^6 leucocytes	
Normal	12	125 ± 26	
Acute uraemia	4	80 ± 14	$p < 0.01$
Chronic uraemia	8	165 ± 17	$p < 0.005$
Alcoholic cirrhosis	8	63 ± 19	$p < 0.001$
Paracetamol necrosis	4	31 ± 15	$p < 0.001$
Obstructive jaundice	6	175 ± 33	$p < 0.01$

Table II. ^{14}C-leucine uptake by leucocytes [12]

		dpm/5 × 10^3 leucocytes	
Normal	10	9,282 ± 421	
Acute uraemia	4	5,925 ± 600	$p < 0.001$
Chronic uraemia	8	8,300 ± 800	$p < 0.05$
Alcoholic cirrhosis	6	7,269 ± 558	$p < 0.001$
Paracetamol necrosis	3	1,964 ± 152	$p < 0.001$
Obstructive jaundice	4	11,200 ± 250	$p < 0.001$

possible impairment in jaundice or uraemia has been made by examining glucose utilisation by the HMP shunt, the ability of cells to take up ^{14}C leucine [12] and their capacity for iodination [7]. MPO and leucocyte esterase were also quantitated, and a study was also made of the effects of potential toxins on the former and on the related enzyme microperoxidase that is an integral part of cytochrome c.

Methods

Leucocytes were isolated from heparinised venous blood by sedimentation of the erythrocytes by addition of 3% dextran and thereafter centrifugation of the plasma at 800 g for 5 min. Contaminating erythrocytes were removed by rapid hypotonic lysis using 5 ml cold 0.2% NaCl for 20 sec followed by the addition of 5 ml 1.6% NaCl. After repeating this procedure the cells were resuspended in appropriate buffer containing 5 mM dextrose. The neutrophil count was always in excess of 85%.

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The cells were suspended in Hanks' neutral salt solution containing 5 mM dextrose. HMP shunt activity was determined with and without stimulation by serum-treated zymosan using flasks containing 3–5 × 10⁶ leucocytes in Hanks solution, 5.0 mM glucose and 0.05 μCi of glucose-1-^{14}C. Incubations were performed for 15, 30, 45 min whilst the evolving $^{14}CO_2$ was adsorbed by an inner chamber containing 0.5 ml 1 N NaOH and 0.5 ml hyamine on a plug, and also for 30 min after termination of the reaction by addition of 60% citric acid. These adsorbent plugs were then dissolved in toluene scintillation medium prior to counting.

In additional experiments in which the effects of bilirubin, and of taurocholate and glycocholate on HMP shunt activity were examined, they were first added to the leucocytes in autologous serum 10 min prior to commencement of the assay by addition of glucose-1-^{14}C. Each was examined at 50 and 25 μM concentration.

Leucine Uptake

100 μl of leucocyte suspension (approx. 5 × 10⁵ cells) was incubated with 100 μl Hanks' solution, to which at time zero was added 50 μl phosphate-buffered saline containing 0.1 μCi ^{14}C-leucine. The reaction was set up in a series of tubes and was stopped at 5, 10, 15 min by the addition of 100 μl cold 10% trichloracetic acid (TCA). The precipitated proteins were then washed and resuspended thrice in cold normal saline containing 1.0 mM unlabelled leucine. Finally each cell button was dissolved in 0.1 M NaOH prior to counting in toluene scintillation fluid.

Leucocyte Iodination Capacity

Cells were resuspended in Kreb's-Ringer phosphate buffer containing 5 mM dextrose and 8 × 10^{-8} M sodium iodide. To 0.2 ml cell suspension (approx. 5 × 10⁵ cells) was added 0.1 ml pooled AB serum and to 'resting tubes' 50 μl plain buffer and to 'stimulated tubes' 50 μl opsonised zymosan. After 15 min preincubation in a shaking water bath at 37 °C, 50 μl of ^{131}I (thiosulphate-free) was added and the tubes were incubated for 30 min. The reaction was terminated by the addition of 0.1 ml of 0.01 M sodium thiosulphate and then 0.2 ml 5% TCA. The tubes were centrifuged to yield the protein deposit, which was then washed thrice by thorough mixing and resuspension in saline. The radioactivity of 'resting' and 'stimulated' tubes was counted in a gammacounter. The former count was subtracted from the latter to give the 'stimulated iodination capacity' [7].

Cells from patients with uraemia or jaundice were studied both in AB normal serum and in their own sera. The effects of the differing sera on cells from normal persons and on those from other clinical entities were also studied. In studies designed to assess the effects of possible toxins and drugs on the leucocyte iodination capacity of normal cells doubling dilutions were made down to infinitesimally small concentrations in a volume of 0.2 ml, and the above volumes of cells and reagents were added for the assay. The effect of each toxin was examined thrice in order to determine the concentration that would reduce iodination by 50%.

MPO Activity

Leucocytes from patients with uraemia or jaundice were counted and were then frozen and

leucocytes of 12 normal persons, 4 patients with acute uraemia, 8 persons with chronic uraemia on dialysis, 8 patients with jaundice due to alcoholic cirrhosis, 4 patients with paracetamol-induced hepatic necrosis and 6 with obstructive jaundice. The normal values were 125 ± 26 nmol ^{14}C-1-glucose oxidised/h, corrected to 5×10^6 leucocytes. Cells from patients with acute uraemia and with alcoholic cirrhosis showed a moderate depression of HMP shunt activity. There was a marked reduction of activity in the leucocytes of the 4 patients with fulminant hepatic failure due to paracetamol necrosis. Leucocytes from 6 patients with obstructive jaundice showed increased HMP shunt oxidation and there was a similar increase in leucocytes from patients with chronic uraemia.

When the effects of bilirubin and bile acids on normal leucocyte HMP shunt activities were examined, it was found that 50 μM bilirubin inhibited HMP shunt activity by 13 ± 3 %. On the other hand, 50 μM taurocholate increased both resting and zymosan-stimulated HMP shunt activity by 70% and 50 μM glycocholate increased these parameters by 35%.

Leucine Uptake by Leucocytes

Table II gives the results of ^{14}C-leucine uptake by 5×10^6 cells from normal persons over 10 min (9,282 ± 421 dpm) as compared with 4 patients with obstructive jaundice, whose cells showed an increased uptake (11,200). There was a marked depression of uptake in acute uraemia and a slight depression in the leucocytes of persons with chronic uraemia. Leucocytes from patients with alcoholic cirrhosis showed moderate depression (mean 7,269) and there was a profound reduction in the white cells of persons with paracetamol-induced necrosis of the liver.

Leucocyte Iodination Capacity

Table III shows the effects of uraemic or jaundiced sera on the iodination capacity of normal leucocytes, together with results for uraemic or jaundiced leucocytes in their own sera. Variations of up to 10% can be caused by resuspending white cells in the sera of other donors but the effect of pooled AB serum is negligible. When normal white cells were suspended in the sera of acutely uraemic patients a 15–20% reduction in iodination capacity occurred. Uraemic white cells in their own sera showed a 15–35% depression compared with normal cells in AB serum. Normal white cells in jaundiced sera showed a 25–30% reduction of their iodination capacity whilst cells from obstructive jaundice patients in their own jaundiced sera showed a 30–35% increase of iodination as compared with normal leucocytes. Jaundiced white cells in uraemic serum showed an inhibition of their iodination.

Effect of Relevant Toxins on Leucocyte Iodination Potential

Table IV lists the concentration of each potential toxin or drug that was required to reduce leucocyte iodination by 50%. The standard uraemic toxins such as urea, creatinine, methylguanidine and *p*-aminohippurate had no effects but indole-3-acetic acid caused 50% inhibition at 50 μg/ml (0.3 mM). Unconjugated bilirubin caused inhibition at only $4 \times 10^{-4} M$ and sodium taurocholate inhibited at 20×10^{-4} M as did cholic acid, but sodium glycocholate was without effect. Phenol-inhibited iodination at a concentration of $5 \times 10^{-3} M$ (0.5 mg/dl) but phenylglucuronide was without effect. Paracetamol, *p*-hydroxyphenylacetic acid, 3 4-dihydroxybenzoic acid and *o*-aminophenol all caused inhibition, as did the anti-inflammatory drugs

Table III. Stimulated iodination capacity of white cells and the effects of pathological sera

Leucocytes	Serum	n	Result nmol/h/10^7 polys	% change	Significance
Normal	Normal	24	60 ± 13		
Normal	uraemia	8	50 ± 8	− 17	$p = 0.05$
Uraemia	uraemia	8	42 ± 10	− 30	$p < 0.01$
Uraemia	OJ	8	46 ± 12	− 25	$p < 0.01$
OJ	normal	8	70 ± 13	+ 15	$p = 0.05$
OJ	OJ	8	80 ± 8	+ 33	$p < 0.01$
Normal	OJ	12	45 ± 10	− 25	$p < 0.01$
OJ	uraemia	6	40 ± 12	− 33	$p < 0.01$

OJ = Obstructive jaundice.

Table IV Toxins and drugs that reduce leucocyte iodination by 50% (concentration × 10^{-5} *M*)

Urea	–	*p*-hydroxyphenylacetic acid	100
Methylguanidine	–	2,5-dihydroxybenzoic acid	100
Creatinine	–	3 4-dihydroxybenzoic acid	100
Hippurates	–	*p*-hydroxybenzoic acid	100
Indole-3-acetic acid	30	aspirin (*in vitro*)	
Phenol	5	sodium salicylate	2
Phenylguanidine	–	mandelic acid	–
Unconjugated bilirubin	4	paracetamol (acetaminophen)	30
Glycocholate	–	*o*-aminophenol	30
Taurocholate	12	phenylbutazone	12
Cholic acid	1	ethanol	500
		ascorbic acid	100

Table V Peroxidase and esterase assay

Category	n	Peroxidase, μmol/10^7 cells	Esterase, μg N/10 cells	
Normal	10	5.1 ± 1 4	32 ± 6	
Acute uraemia	4	3.3 ± 0.8 $p < 0.05$	23 ± 14	n.s.
Chronic uraemia	10	13.9 ± 3.6 $p < 0.001$	23 ± 10	n.s.
Alcoholic cirrhosis	6	4.6 ± 0.7	20 ± 11 $p < 0.05$	
	7	3.3 ± 0.5 $p < 0.01$		
Paracetamol necrosis	4	2.8 ± 1.8 $p < 0.05$	25 ± 9	n.s.
Obstructive jaundice	8	14 7 ± 4.8 $p < 0.001$	64 ± 16 $p < 0.01$	

leucocytes of 12 normal persons, 4 patients with acute uraemia, 8 persons with chronic uraemia on dialysis, 8 patients with jaundice due to alcoholic cirrhosis, 4 patients with paracetamol-induced hepatic necrosis and 6 with obstructive jaundice. The normal values were 125 ± 26 nmol ^{14}C 1-glucose oxidised/h, corrected to 5 × 10^{6} leucocytes. Cells from patients with acute uraemia and with alcoholic cirrhosis showed a moderate depression of HMP shunt activity. There was a marked reduction of activity in the leucocytes of the 4 patients with fulminant hepatic failure due to paracetamol necrosis. Leucocytes from 6 patients with obstructive jaundice showed increased HMP shunt oxidation and there was a similar increase in leucocytes from patients with chronic uraemia.

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phenylbutazone and sodium salicylate. Aspirin had no *in vitro* action yet studies on 6 medical students showed that after ingestion of 600 mg aspirin there was a 20% reduction of their leucocyte iodination capacity Presumably this reflects the action of a metabolite. Total leucocyte counts did not change Ethanol caused 50% inhibition of iodination at the relatively low concentration of 0.5 mM (25 mg/dl) and ascorbic acid at 0 1 mM (20 mg/dl)

MPO and Esterase Activity (table V)

MPO activity was reduced in paracetamol poisoning and in acute uraemia. Half of the patients with alcoholic cirrhosis had low values. Patients with obstructive jaundice and with chronic uraemia had elevated MPO levels. Esterase activity was reduced in alcoholic cirrhosis and perhaps in acute and chronic uraemia, whilst values were certainly elevated in the leucocytes of patients with obstructive jaundice.

Effect of Toxins on MPO Microperoxidase and NADPH Oxidase

As will be seen from table VI the standard uraemic toxins did not affect MPO but the various phenols caused inhibition. Bilirubin and the bile acids caused half maximal inhibition at modest concentrations. All agents caused uncompetitive inhibition meaning that the maximum capacity of the enzyme (the V_{max}) was reduced and at the same time the concentration of substrate to cause half maximal activation (the Michaelis constant) was increased. Almost identical reagents caused inhibition of microperoxidase

A homogenate of normal leucocytes gave an NADPH oxidase activity of 16 nmol/min/mg and this was reduced to 50% by 1.5 μM unconjugated bilirubin and by 25 μM taurocholate or glycocholate. The activity was also reduced by 100 μM ascorbic acid Conversely 100 μM phenol increased the NADPH oxidase activity by 50%

Table VI. Concentrations of toxin required to [illegible] 50% enzyme inhibition (concentrations × 10^{-6} M)

	Myeloperoxidase	Microperoxidase	NADPH oxidase
Urea	–	–	–
Creatinine	–	–	–
Methylguanidine	500	100	–
Phenol	100	20	10[1]
Bilirubin	8	8	0.15
Cholic acid	20	1	2.5
Taurocholate	20	2	2.5
p-OH-phenylacetic	300	30	–
p-OH-benzoic acid	–	10	–
Paracetamol	80	2	–
3 4-dihydroxybenzoic	30	25	–
Phenylglucuronide	–	15	–
Acetylsalicylic acid	30	20	–
Ascorbic acid	100	50	10

1 Stimulation occurred.

Discussion

The susceptibility to infection of patients with uraemia [13] or certain types of jaundice [4] varies This study has sought to analyse possible metabolic defects and hence the action of toxins that might be relevant. The results do indicate how defects in leucocyte function might arise. There is certainly a defect in HMP shunt activity in leucocytes from patients with fulminant hepatic failure due to paracetamol poisoning [1] and in acute uraemia and some patients with alcoholic cirrhosis [16] Conversely there can be metabolic adaptation because

HMP shunt activity is increased in chronic uraemia and in leucocytes of patients with obstructive jaundice. In paracetamol-induced necrosis of the liver in addition to the HMP shunt defect, there is a depression of ^{14}C leucine uptake (table II) and a depression of MPO activity We have found that unconjugated bilirubin lowers HMP shunt activity whilst the bile acids increase it. These toxins depress leucocyte iodination, whilst the standard uraemic toxins do not (table IV) However phenols and phenolic acids do accumulate in paracetamol-induced hepatic coma, because the glucuronidation and sulphation mechanisms are then inactive. As shown in tables IV and VI, they depress leucocyte iodination and the activity of MPO

There is good evidence that MPO is vital for microbicidal activities [3, 17] Our data show that MPO can be reduced in acute uraemia or in some cases of alcoholic cirrhosis, but conversely its activity is increased in leucocytes of patients with chronic uraemia or obstructive jaundice. MPO is not the critical factor in initiating the respiratory burst [18] Rather this is due to NADPH oxidase whose presence in cell membranes reflects it vital rôle. However it is to be noted that the NADPH oxidase that used in our assays was almost certainly that derived from neutrophilic granules because of the mode of preparation [14] Its properties are the same.

In the leucocytes of patients with alcoholic cirrhosis there was a reduction of HMP shunt activity but not of leucine utilisation. MPO was lowered in half of these patients and esterase activity was subnormal in all. The latter finding indicates a susceptibility to endotoxaemia because esterases help endotoxin inactivation. Since ethanol has been shown to inhibit leucocyte iodination (table IV), so suppression by acute alcoholic intoxication seems likely The biochemical action of alcohol in creating excess of NADPH and NADH could be relevant, for excess of NADPH will suppress glucose-6-phosphate dehydrogenase activity and thereby HMP shunt glucose oxidation [8]

It is of interest that in patients with obstructive jaundice and a liability to cholangitis both HMP shunt activity and utilisation of ^{14}C-leucine for protein synthesis can be increased. Leucocytes that are activated in this way are found in severe infections [10] Our results indicate that increased HMP shunt activity might be due to the effects of bile acids. The leucocytes of patients with obstructive jaundice and those with chronic uraemia showed increased peroxidase activity Thus it is evident that enzymes can undergo an adaptive increase when there is a continual threat of infection in nondebilitated patients.

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phenylbutazone and sodium salicylate. Aspirin had no *in vitro* action, yet studies on 6 medical students showed that after ingestion of 600 mg aspirin there was a 20% reduction of their leucocyte iodination capacity. Presumably this reflects the action of a metabolite. Total leucocyte counts did not change. Ethanol caused 50% inhibition of iodination at the relatively low concentration of 0.5 m*M* (25 mg/dl) and ascorbic acid at 0.1 m*M* (20 mg/dl).

MPO and Esterase Activity (table V)

MPO activity was reduced in paracetamol poisoning and in acute uraemia. Half of the patients with alcoholic cirrhosis had low values. Patients with obstructive jaundice and with chronic uraemia had elevated MPO levels. Esterase activity was reduced in alcoholic cirrhosis and perhaps in acute and chronic uraemia, whilst values were certainly elevated in the leucocytes of patients with obstructive jaundice.

Effect of Toxins on MPO Microperoxidase and NADPH Oxidase

As will be seen from table VI the standard uraemic toxins did not affect MPO but the various phenols caused inhibition. Bilirubin and the bile acids caused half maximal inhibition at modest concentrations. All agents caused uncompetitive inhibition, meaning that the maximum capacity of the enzyme (the V_{max}) was reduced and at the same time the concentration of substrate to cause half maximal activation (the Michaelis constant) was increased. Almost identical reagents caused inhibition of microperoxidase.

A homogenate of normal leucocytes gave an NADPH oxidase activity of 16 nmol/min/mg and this was reduced to 50% by 1.5 μM unconjugated bilirubin and by 25 μM taurocholate or glycocholate. The activity was also reduced by 100 μM ascorbic acid. Conversely 100 μM phenol increased the NADPH oxidase activity by 50%.

Table VI. Concentrations of toxin required to cause 50% enzyme inhibition (concentrations × 10^{-6} *M*)

	Myeloperoxidase	Microperoxidase	NADPH oxidase
Urea	–	–	–
Creatinine	–	–	–
Methylguanidine	500	100	–
Phenol	100	20	10[1]
Bilirubin	8	8	0.15
Cholic acid	20	1	2.5
Taurocholate	20	2	2.5
p-OH-phenylacetic	300	30	–
p-OH-benzoic acid	–	10	–
Paracetamol	80	2	–
3,4-dihydroxybenzoic	30	25	–
Phenylglucuronide	–	15	–
Acetylsalicylic acid	30	20	–
Ascorbic acid	100	50	10

[1] Stimulation occurred.

Discussion

The susceptibility to infection of patients with uraemia [13] or certain types of jaundice [4] varies. This study has sought to analyse possible metabolic defects and hence the action of toxins that might be relevant. The results do indicate how defects in leucocyte function might arise. There is certainly a defect in HMP shunt activity in leucocytes from patients with fulminant hepatic failure due to paracetamol poisoning [1] and in acute uraemia and some patients with alcoholic cirrhosis [16]. Conversely there can be metabolic adaptation because

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The Volume of Plasma and Erythrocytes in Individual Bones and in the Spleen of Mice under Physiological Conditions and with Acute Radiation-Induced Atrophy of the Haemopoietic Tissue

J. Vácha, J. Holá, J. Dungel and V. Znojil

Institute of Biophysics of the Czechoslovak Academy of Sciences, Brno, Czechoslovakia

Key Words. Bone marrow · Distribution · Erythrocytes · Haem iron · Haemopoietic organs · Plasma · Radiation · Skeleton · Spleen

Abstract. Using labelled erythrocytes and human serum albumin, the volume of circulating erythrocytes and plasma was determined in the spleen and individual parts of the skeleton of mice under physiological conditions and 48 h after X-irradiation with a dose of 2,87 Gy. The results are significant for the interpretation of the method of incorporation of labelled iron into haemopoietic organs.

Introduction

The method of incorporation of isotopically labelled iron, frequently used for determining the intensity of erythropoiesis and changes in it, gives markedly different results according to the region measured, e.g. from one bone to another [4]. One of the reasons for this is the different proportion of circulating blood in the organs investigated, for both plasma and erythrocytes (RBC) can contain significant amounts of radioactivity, especially in situations where erythropoiesis is suppressed, and its contribution to the content of the label in the organ reduced. Knowledge of the volume of plasma and RBC in the various parts of the haemopoietic system would allow appropriate corrections to be made.

Methods

Animals, Irradiation

Male mice of the inbred strain C57BL/10ScSnPh were used; the diet used was DOS from Velaz (Prague); it contained 320.8 ± 29.5 mg Fe/kg. The animals had free access to food and water. Some of the animals remained unirradiated, some of them were whole-body X-irradiated with 300 R (dose 2.87 Gy). According to our experience, this dose causes a fall in the intensity of erythropoiesis to less than 1/5 of the initial level in 48 h, i.e. at the time when blood volume measurements were made. The irradiation was carried out on a therapeutic TUR apparatus (GDR), dosimetry was performed using a Radocon II instrument (Victoreen).

Determination of the Volume of Circulating RBC in Organs

Both non-irradiated and irradiated animals aged 14.8 ± 0.5 weeks were used, body mass was

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E. N Wardle, The Liver Unit,
King's College Hospital, Denmark Hill,
London SE5 (England)

Table I. Volume of RBC (frozen animals) and plasma (bled animals) in individual parts of the haemopoietic system (μl) of non-irradiated mice

Organ	RBC volume, μl = 22	Plasma volume, μl n = 24	Bone mass mg
Whole body	(739 ± 13)[a]	1,143 ± 25	
Spleen	10.579 ± 0.420 (6.259 ± 0.379)[a]	6.572 ± 0.229 (10.274 ± 1.285)[a]	
Skull	15.580 ± 0.816	22.851 ± 0.641	235.03 ± 4.71
Mandible	3.434 ± 0.177	5.119 ± 0.116	85.78 ± 0.75
Vertebrae, cervical to sacral	17.334 ± 0.556	35.077 ± 1.424	268.23 ± 4.30
Vertebrae, tail	3.538 ± 0.794	9.574 ± 0.758	156.57 ± 3.11
Sternum	1.544 ± 0.078	4.708 ± 0.355	19.53 ± 0.47
Ribs	4.494 ± 0.251	7.306 ± 0.571	64.13 ± 1.27
Scapulae	1.048 ± 0.076	2.373 ± 0.077	27.38 ± 0.44
Clavicles	0.271 ± 0.028	0.589 ± 0.056	5.76 ± 0.12
Humeri	1.492 ± 0.070	4.106 ± 0.098	42.53 ± 0.54
Radii, ulnae	0.532 ± 0.019	1.665 ± 0.052	33.27 ± 0.37
Forefeet	0.371 ± 0.044	1.620 ± 0.154	22.24 ± 0.62
Pelvis	3.372 ± 0.140	7.689 ± 0.516	73.89 ± 0.97
Femora	3.484 ± 0.141	7.595 ± 0.233	92.56 ± 1.26
Tibiae, fibulae	2.601 ± 0.066	6.162 ± 0.193	85.76 ± 1.00
Hindfeet	1.067 ± 0.070	3.576 ± 0.303	78.28 ± 1.50
Whole skeleton	60.242 ± 1.891	120.06 ± 3.19	1,286.11 ± 0.01

= Number of animals.
For non-irradiated and irradiated animals.
Results for bled animals.
Results for frozen animals.

Results

Non-Irradiated Animals

Table I gives the volumes of RBC for non-irradiated mice only for those of the animals that were sacrificed by freezing, since compared with the frozen animals, the spleen and bone marrow values of the bled animals proved to be highly significantly lower ($p < 0.005$- only the values given in parentheses do not refer to animals sacrificed by freezing; the value for the spleen represents the maximum estimate of RBC volume on the basis of haem iron content in this organ – in bled animals see below). Table I also reports data for plasma volumes only for those of the animals which were bled (except for the value given in parentheses for the spleen, which is for frozen animals). In frozen animals, the plasma values for the bone marrow were significantly lower than in the bled animals, $p < 0.025$ only in the spleen was it the other way round, $p < 0.05$

24.5 ± 1.4 g for the non-irradiated animals and 30.6 ± 0.6 g for irradiated mice. The RBC of the donors were finely rinsed in an excess of buffer (NaCl, KCl, Na_2HPO_4, KH_2PO_4) 0.2–1.0 ml commercial Na_2CrO_4 solution labelled with [51]Cr (*containing 7.05 Bq–2.28 Bq/ml, specific activity* 3.40 Bq–1.60 Bq /μg), previously diluted to a volume of 2.3–3 ml with physiological saline, was slowly added to 5–8 ml of packed RBC. The suspension was incubated for 30 min at room temperature, washed 3–4 times in the above buffer and, after 20 min centrifugation at 1,400 g injected into the tail veins at a dose of 100 μl. Blood samples were taken after 30 or 60 min (with no effect on the results), microhaematocrit was determined and blood samples of 200 μl in volume were used for the measurement of radioactivity (Automatic Gamma Well Counting System, Nuclear Chicago). At the same interval, a further group of animals was placed under light ether anaesthesia and immersed in liquid nitrogen. Immediately after bleeding or freezing, the spleens of all animals were excised and their radioactivity measured. The skeletons of the animals were completely cleaned of soft tissue through the saprophagic activity of the insect *Dermestes vulpinus* Fbr., bred as a laboratory colony: the skeletons were divided into 15 bone groups (tables I, II) and their radioactivity measured. After drying to constant mass (several days at room temperature), the individual bones were weighed.

Determination of Plasma Volume in Organs

Both non-irradiated and irradiated animals aged 12.4 ± 0.1 weeks were used, body mass was 27.3 ± 0.5 g for the non-irradiated animals and 26.9 ± 0.8 g for the irradiated mice. The animals received 100 μl solution of human serum albumin, labelled with [131]I or [125]I (5.74 Bq/ml or 2.98 Bq/ml), into the tail veins; after 15 min, the animals were bled and plasma samples of 100–300 μl in volume were used for the measurement of radioactivity. A further group of animals was sacrificed by immersion in liquid nitrogen. The processing of the organs (spleens and skeletons) was carried out as described for the previous experiments.

Measurement of Haem Iron Content in Organs

The mice used (n = 24) were 11.0 ± 0.4 weeks old, body mass was 24.5 ± 0.5 g. Some of the animals (n = 10) were irradiated with the described dose, the irradiated and non-irradiated animals bled after 48 h, the skeletons freed of soft tissue by *D. vulpinus* and the individual bones and the spleens subjected to haem extraction using acid ethylacetate [for details of this original method, see *Vácha et al.*, 7]; the amount of haem was then converted into the amount of iron bound to it. The concentration of haemoglobin (haemiglobin cyanide method) and haematocrit in the blood from the axillary vessels were determined.

Numerical Processing

The total volume of circulating RBC in the body was calculated according to *Vácha* [6]. *The volume of circulating RBC in the organ* was calculated according to the equation.

$$RBCV = \frac{OA \cdot VW \cdot PCV \cdot 0.965}{AW \cdot 100}$$

where OA is the radioactivity of the organ, VW the volume of blood withdrawn, PCV the sample packed cell volume (in %), 0.965 the correction factor for retained plasma, AW the activity of the blood sample withdrawn. There was no correction for the presence of applied RBC, since 15 min after the application of labelled RBC the venous haematocrit had reverted to the control value (apparently as a result of the dilution of the blood), and it can be assumed that the total volume of blood in the bone cavities remains unchanged in spite of the overall increase in circulating blood volume. *The total volume of plasma in the body* was determined according to the equation.

$$PV = \frac{VW_p \cdot AA}{AW_p \cdot 1.08}$$

where VW_p is the volume of plasma withdrawn, AA_p the radioactivity applied, and AW_p the radioactivity of the plasma sample withdrawn. The deduced factor of 1.08 [3, 5] represents the loss of labelled serum albumin from the plasma. *The plasma volume in the organ* (PV_o) was calculated as the ratio between the radioactivity of the organ and the radioactivity of a unit volume of withdrawn plasma.

The results are expressed as mean ± SEM; the statistical significance of differences was evaluated by means of a t test.

Table II. Volume of RBC (frozen animals) and plasma (bled animals) in individual parts of the haemopoietic system for mice irradiated with a whole-body dose of 2.87 Gy 48 h previously

Organ	RBC volume, μl n = 6	Plasma volume, μl = 8
Whole body	(664 ± 31)	1,247 ± 39
Spleen	6.742 ± 0.352 (2.652 ± 0 092)	3 451 ± 0.296 (7.945 ± 1.994)
Skull	17 654 ± 1.064	25.892 ± 1.317
Mandible	3.156 ± 0.078	4 702 ± 0.386
Vertebrae, cervical to sacral	26.502 ± 0 665	57 792 ± 2.341
Vertebrae, tail	2.526 ± 0 376	9.046 ± 0.889
Sternum	2.404 ± 0.138	8.156 ± 0.455
Ribs	5.279 ± 0.354	14.191 ± 0.448
Scapulae	1.844 ± 0.117	4.611 ± 0.138
Clavicles	0.404 ± 0.060	0 647 ± 0.165
Humeri	2.891 ± 0 051	9 067 ± 0.187
Radii, ulnae	0.746 ± 0 025	2.817 ± 0.160
Forefeet	0 476 ± 0 068	1.473 ± 0.148
Pelvis	5.213 ± 0.739	18.497 ± 1.113
Femora	6.886 ± 0.320	17 761 ± 0.442
Tibiae, fibulae	4.677 ± 0.144	13.822 ± 0 992
Hindfeet	1.360 ± 0.137	3.399 ± 0.212
Whole skeleton	82 016 ± 1.201	191.87 ± 5.85

= Number of animals.
Results for bled animals.
Results for frozen animals.

– even more so than those for non-irradiated mice – were obtained when haem iron was calculated from the volume of RBC, these figures are 6.17 ± 0.39 μg Fe for the spleen and 70.0 ± 3.5 μg Fe for bone marrow Since the same considerations apply to the differences as in the case of non-irradiated animals, it was convenient to estimate the upper possible limit of RBC volume in the spleen of irradiated (bled) animals using the value obtained for haem iron this estimate is 2.652 ± 0.092 μl RBC.

Discussion

The agreement between the haem iron content obtained for RBC and for organ extract is very good in the case of non-irradiated marrow But the value of RBC haem iron for the spleen is significantly higher than that obtained by extraction (especially in the post-irradiation state), which probably results – in addition to the different methods of obtaining material – from a certain fraction of transfused labelled RBC being trapped in this organ [see e.g. *Anosa*,

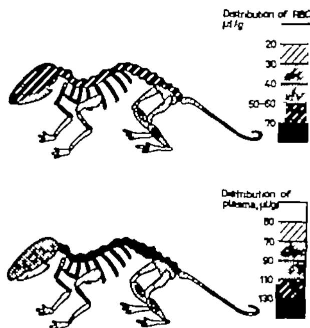


Fig. 1. Schematic depiction of the distribution of RBC and plasma in the skeleton of intact mice per unit mass of individual bones (µl/g tissue)

The spleen mass of the mice used to determine RBC content was 84 6 ± 6 1 mg· in the mice used to determine plasma con tent, it was 86 6 ± 3 8 mg.

The volumes of RBC and plasma in org ans calculated per unit mass of the organ are given in figure 1 which shows that the highest amount of RBC and plasma per unit mass is concentrated in the axial skeleton of the mouse (and especially in the thoracic bones) and that the content of the two com ponents increases in the limbs in a proximal direction.

The amount of haem iron in the spleen of non-irradiated animals (n = 14) was 7 01 ± 0 40 µg· in the skeleton it was 63.8 ± 2.8 µg. The concentration of haemoglobin in blood from the axillary vessels was 16.32 ± 0 12 g/100 ml, with a haematocrit of 48.92 ± 0.25 A known iron content of 0.336% by mass in the haemoglobin, means that 1 µl RBC contains 1 12 ± 0 01 µg haemoglobin bound Fe If on the basis of this figure, we convert the volume of RBC into the amount of haem iron we get the value of 9.39 ± 0.39 µg in the spleen and 49 4 ± 2.3 µg in the skeleton. Both these figures differ in a highly significant manner ($p < 0\ 001$) from values ascertained by direct extraction of haem iron – for the skeleton, the figure is lower for the spleen, it is higher than the values measured. While the difference in the case of the skeleton is apparently the result of the presence of haem iron in erythroblasts, the difference in the case of the spleen can be explained only as resulting from an overestimation of the RBC volume by the method used. It is therefore useful to estimate the volume of RBC in the spleen on the basis of the haem iron content the resulting figure is 6.259 ± 0.379 µl RBC, which is the upper estimate of RBC volume in the spleen (of bled animals) since the haem iron contained in ery throblasts is here neglected.

Irradiated Animals

The results for irradiated animals are ar ranged as those for non-irradiated animals and are reported in table II The spleen mass of mice used for the determination of RBC content was 38.3 ± 0 6 mg· in the mice used for determining the plasma volume, it was 36 0 ± 2.8 mg.

The post irradiation changes in the RBC and plasma volume (expressed in percent of non-irradiated animals) in individual re gions of the skeleton are highly significantly correlated (Spearman s coefficient of rank correlation $r = 0\ 744$ $p < 0\ 001$ $n = 15$)

The amount of haem iron in the spleen of irradiated animals (n = 10) was 2.97 ± 0 10 µg· in the skeleton it was 154 45 ± 8.9 µg. Highly significantly different figures

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Jiří Vácha, MUDr CSc, Institute of Biophysics ČSAV Královopolská 135 (Czechoslovakia)

1] Thus, while the values for RBC volume obtained with the bone marrow can be considered relatively reliable, the value which best corresponds to reality in the case of the spleen is more likely to be the one calculated from the content of extractable haem iron (the value given in brackets in table I and II)

As far as plasma volume in the organs is concerned, the results obtained for the bone marrow in bled animals may be considered subject to a lower error (in the sense of a loss of plasma content) though of unknown magnitude, than the results obtained in frozen material. In the case of the spleen it is probably preferable to take the higher values obtained with frozen material (the value given in parentheses in tables I and II) as being more accurate, apparently in connection with the contraction of this organ on acute blood loss. While the values for plasma volume in bone marrow of bled animals shown in tables I and II are subject to error only from the possible escape of a certain amount of plasma, all RBC and plasma values listed for the spleen in tables I and II must be regarded as being less reliable due to a number of complicating factors in respect of this organ (contractility phagocytic and immune activity)

The changes in the erythrocyte and plasmatic volume in the phase of post-irradiation atrophy of haemopoietic tissue took place in parallel in both components they consisted of a slight to very marked increase in volume (up to more than double the pre irradiation content) It can be assumed with certainty that this flow of blood into individual bones is related to the known phenomenon of post-irradiation extravasation through damaged endothelia of the marrow sinuses [e.g. 2] The fall in blood volume in the spleen after irradiation is evidently associated with its overall loss of mass and therefore of volume, which does not occur with bones.

It follows from the results of the extraction of haem iron that, while there is reasonable agreement between the contents of RBC and haem iron in bones under physiological conditions, after irradiation the content of haem iron rises much more sharply than that of circulating RBC. This circumstance would suggest that a considerable proportion of the RBC taking part in post-irradiation extravasation has already been removed from circulation and does not mix with the injected labelled RBC. A histological phenomenon correlated with the discrepancy mentioned appears to be the clotting of blood observed both intravascularly and at sites of parenchymal haemorrhages [2]

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6 Vácha, J Blood volume in inbred strain BALB/c, CBA/J and C57BL/10 mice deter

patients, each patient received 60 mg of KI orally and then 30 mg on each subsequent day in order to block thyroidal uptake of isotope. The initial dose of ^{125}I-labelled human fibrinogen (Radiochemical Centre Amersham) was injected intravenously as soon as possible after admission and lower-limb scanning was carried out approximately 4 h later according to the method of *Kakkar et al.* [3]. This was repeated daily and leg vein thrombosis was diagnosed only if there was difference in the count of 20% between adjacent positions on the same leg or similar positions on the two legs.

In accordance with the current policies of the different medical teams in this hospital, 27 patients were treated from the time of admission with subcutaneous heparin 5,000 U b.d. as prophylaxis against venous thrombosis [4, 5], and 7 patients did not receive heparin.

Blood was taken for BTG estimation at the time of admission and repeated daily. Venepuncture was carried out by only three individuals throughout the study in order to minimize error introduced by varying techniques. Blood was taken by clean puncture, without venous occlusion and transported immediately to the laboratory in tubes containing EDTA and theophylline in ice for separation of plasma. Plasma BTG was estimated by standard radio-immune assay (Amersham). Neither the technician responsible for leg scanning and subsequent interpretation of the tracings nor the haematologist conducting the BTG assay had any information concerning the clinical status of the patients under study. In those patients with leg scans consistent with thrombosis, lower limb venography was carried out when feasible.

Results

7 of the 34 patients with proven myocardial infarction developed positive ^{125}I fibrinogen scans. In 3 of these patients, lower-limb venography was carried out and 2 had radiologically apparent clots. Of the 27 patients treated with prophylactic subcutaneous heparin, 5 developed positive scans. 2 of the 7 patients who did not receive heparin also had positive scans.

6 of the 7 patients with positive scans were non-smokers whereas there were equal numbers of smokers and non-smokers in the total group of 34 patients.

5 patients with thrombosis first developed an abnormal scan on the 3rd day after infarction. The 2 remaining patients developed abnormal scans on days 2 and 4 respectively.

The normal range for plasma BTG in adults is 15–30 U. In only 2 patients was there an unequivocally raised BTG level on the same day as the fibrinogen scan first became abnormal and in 6 of the 7 patients there was no clear-cut rise in the BTG level on this day compared to previous days (table I).

In the majority of patients with myocardial infarction, the BTG was raised on day 1 (table II), but there was no significant difference in level between patients with anterior and inferior infarction ($p > 0.1$). There was a trend to higher values of BTG in patients with a CPK greater than 500 compared to those with a CPK less than 500 but this was not significant ($p > 0.1$). There was no difference in the 1st-day BTG between those patients who subsequently developed DVT and the remainder (p

Table I. Serial BTG in patients with thrombosis

Patient	Day 1	2	3	4	5	6	7
1	59	32	41	36	61	64	72
2	140	72	23[1]	33	70	48	95
3	49	56	21	62	38	33	33
4	74	34	88[1]	120	66	109	204
5	38	42	34[1]	40	90	75	77
6	30	36[1]	50	41			
7	80		85	78			

[1] Day on which fibrinogen scan first became positive.

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Beta-Thromboglobulin Levels in Relation to Myocardial Infarction – Preliminary Observations

J P Simmonds B P O Malley M R Maskill J F O Connor and D J Burridge

Departments of Clinical Pharmacology Haematology and Medical Physics, Leicester Royal Infirmary Leicester

Key Words. Myocardial infarction Smoking β Thromboglobulin Deep-venous thrombosis

Abstract. 34 patients with myocardial infarction were studied with daily measurements of β-thromboglobulin (BTG) and ^{125}I fibrinogen scanning in order to detect deep-venous thrombosis (DVT) Serial levels of BTG were unhelpful in the early detection of this condition, which occurred in 7 of the patients studied. 5 of the patients with DVT had received prophylactic heparin and 6 of them were non-smokers. This latter observation is in support of previous studies suggesting an increased incidence of DVT after myocardial infarction in non-smokers as compared to smokers.

Introduction

β-Thromboglobulin (BTG) is a platelet specific protein released during platelet aggregation. Elevated plasma concentrations of BTG have been reported in deep-venous thrombosis (DVT) [1] and, consequently it has been suggested that their measurement may prove a useful screening test for the presence of thrombus. Elevated plasma BTG has also been reported in myocardial infarction [2] and DVT is a well-recognised complication of the post-infarction period.

We, therefore, studied patients admitted to hospital with suspected myocardial infarction in order to determine whether daily plasma BTG estimations had any contribution to make towards the early detection of venous thrombosis.

Patients and Methods

Informed consent was obtained from 34 consecutive patients admitted to the Leicester Royal Infirmary with suspected myocardial infarction, subsequently confirmed by serial ECGs and serum enzyme levels.

The patients comprised 32 men and 2 women, ranging from 42 to 79 years of age. Of the 34 patients with definite myocardial infarction, 17 had anterior and 12 had inferior involvement. All patients were investigated over 7 days, there being a 48-hour period of bed rest followed by gradual mobilisation until discharge on the 7th or 8th day Following the initial clerking and baseline investi-

Thus daily estimations of serum BTG were not found to be helpful in the early detection of DVT after myocardial infarction. However an elevated serum BTG would seem to be a feature of uncomplicated myocardial infarction, and to some extent, reflects infarct size.

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Dr J. P. Simmonds, MRCP
Senior Medical Registrar
Leicester Royal Infirmary Infirmary Square,
Leicester LE1 5WW (England)

Table II. Mean BTG on day of admission in all patients

	Number	Mean BTG ± SD
Anterior infarcts	17	65.2 ± 57.9
Inferior infarcts	12	84 7 ± 65.9
Unknown site	5	79.5 ± 38
CPK > 500	21	78.2 ± 56.4
CPK < 500	13	44 0 ± 23.9
Patients who developed thrombosis	7	70.0 ± 34.9
Smokers	17	70.7 ± 39 1
Non-smokers	17	63.9 ± 56.1

> 0.5) and also no difference between smokers and non-smokers ($p > 0.5$)

Discussion

In this preliminary study plasma BTG estimations were of no value in diagnosing or predicting venous thrombosis, occurring in patients with myocardial infarction. This contrasts with the findings of *Ludlam et al* [1] where unequivocally elevated BTG levels were found in all patients presenting with DVT thus prompting their suggestion that BTG estimation might prove a useful screening test for venous occlusion. However many of their patients differed from ours in having clinically evident thrombosis.

Denham et al [6] found rising values of BTG in a small number of patients with myocardial infarction, who subsequently developed thrombo-embolic complications they suggested that a high plasma BTG on admission coupled with a negative fibrinogen scan was highly suggestive of mural thrombosis, thus calling for anti-coagulation. However in our series, 24 of 34 patients with myocardial infarction had an elevated BTG on the 1st day along with a negative fibrinogen scan and none of these developed manifestations of systemic embolism. Consequently the 1st day elevation of BTG may be a feature of myocardial infarction *per se.*

Evidence to date concerning the value of subcutaneous heparin in preventing DVT has been conflicting but for the most part compatible with effective prophylaxis. *Handley* [7] did not document a prophylactic role for subcutaneous heparin but a criticism levelled at his work was his failure to state the timing of heparin instillation in relation to the onset of chest pain. *Warlow et al.* [4] suggested heparin administration within 12 h of infarction was probably necessary for effective prophylaxis. Inspite of the policy of most medical teams in our hospital that subcutaneous heparin be given immediately on admission, in our series a variable delay often in excess of 12 h was frequently in evidence, and presumably this could account for the occurrence of thrombosis in patients treated with prophylactic subcutaneous heparin, but unfortunately numbers were too small to analyse further

It is interesting to note that of the 7 patients with thrombosis, 6 were non-smokers, despite there being equal numbers of smokers and non-smokers in the whole group, and this is in accordance with the findings of *Emerson and Marks* [8] who reported an increased incidence of thrombosis in non-smokers after myocardial infarction. The reason for this rather surprising finding is uncertain and suggested explanations have included a possible beneficial acute effect on coagulation of stopping smoking. It has also been suggested that smokers, who abruptly stop smoking on admission to a coronary care unit are agitated and move their legs more frequently thus reducing the risk of developing venous thrombosis.

Thus daily estimations of serum BTG were not found to be helpful in the early detection of DVT after myocardial infarction. However an elevated serum BTG would seem to be a feature of uncomplicated myocardial infarction, and to some extent, reflects infarct size.

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Dr. J. P. Simmonds, MRCP
Senior Medical Registrar
Leicester Royal Infirmary Infirmary Square,
Leicester LE1 5WW (England)

Correspondence

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Treatment of Multiple Myeloma with Vincristine

To the Editor

The only literature data on vincristine (VCR) *monochemotherapy in multiple myeloma* are those of *Alberts et al* [1] who successfully used it in stabilizing melphalan (MPH)-induced remissions. More frequently VCR has been associated with two or more alkylating agents for induction therapy *Such protocols have achieved* higher response rates as compared with similar ones without VCR [2]

Table I. Response to VCR and to the association of VCR with PTC in 12 courses of therapy administered to 8 patients with multiple myeloma

Therapy	Observation			Time of diagnosis	Time of therapy	Phase of disease	M-component, g/dl		
							BT	AT	Δ%
VCR 2 mg/7–15 days × 4–5 doses	1	Scu. P.,	G κ	Jun. '75	Aug. '75	CR	1.3	1.2	− 8
	2	Cas. I.,	G λ	Jan. '73	Jul. '76	PR	3 6	3.2	− 12
	3	Mon. E.,	A λ	Oct. '74	May '75	PR	2.2	1.5	− 30
	4	Mon. E.,	A λ	Oct. '74	Aug. '76	CR	1.5	1.5	0
	5	Bal. M	G κ	Feb. '74	Apr '75	PR	1.8	1.2	− 34
	6	Bal. M.,	G κ	Feb. '74	Aug. '76	relapse	2.5	1 6	− 36
	7	Mon. E.,	A λ	Oct. '74	Jun. '78	relapse	2.0	1.2	− 38
	8	Pis. L.,	G κ	Jan. '72	Apr '75	relapse	_.8	4 1	+ 45
	9	Feb. L.,	A κ[1]	Apr '74	May '78	relapse	4 4	3 6	− 18
PTC (40–60 mg/48 h × 5–8 doses) + VCR (1–2 mg on day 1)	10	Feb. L.,	A κ[1]	Apr '74	Dec. '78	relapse	5 4	3.5	− 36
	11	Fil. V	λ	Jun. '75	Dec. '78	relapse	512	378	− 26
	12	Zor P.,	A κ	Mar '75	Oct. '78	relapse	4 0	2.2	− 45

BMPC = Bone marrow plasma cells BT AT = before and after therapy Δ% = percentage change PR, CR = partial and complete response NR = no response.

Patient with plasma cell leukemia.

1 Plasma cells = 69% BT and 2% AT

8 Transfused.

4 U/dl.

Since 1975 we have used VCR in the treatment of multiple myeloma. At first we administered this drug as the only cytostatic with the aim of consolidating partial or complete responses obtained with Peptichemio (PTC) or of treating relapses of the disease when cytopenia and/or alkylating resistance discouraged the use of these drugs. Subsequently we associated VCR on the first day of PTC courses to induce remission in advanced relapsing myelomas resistant to MPH and/or cyclophosphamide. Our overall experience concerns 12 courses of therapy administered to 8 patients (table I). Their effect was evaluated from the changes of the level of the serum M component and of other hematochemical parameters (hemoglobin, blood urea nitrogen, calcium, albumin) as well as from the survey of skeletal X rays and of bone marrow plasmocytomatous infiltrate. Our criteria are detailed elsewhere [3].

BMPC, %		WBC 10^9/l		Platelets 10^9/l		Hb g/dl	
BT	AT	BT	AT	BT	AT	BT	AT
21	16	10.0	9.5	225	220	12.7	15.0
48		2.7	5.1	60	170	9.8	11.4
12		3.6	5.2	70	200	13.4	13.6
		1.8	8.6	85	140	13.5	14.6
25	10	4.1	7.6	105	195	10.1	12.1
38	31	4.1	5.1	75	110	11.0	13.4
16		3.2	4.7	100	180	13.0	14.5
23		3.0	4.9	58	95	11.1	11.8
92	64	1.0	5.0[a]	25	165	7.3	12.6[a]
95		6.4	2.9	97	53	11.0	12.0
70		6.4	5.1	60	35	13.3	13.0
90	53	3.1	1.9	107	65	8.6	14.0[a]

VCR monochemotherapy has given satisfactory results. PTC-induced remissions (observations 1–5), which are in themselves transient [3] were maintained (observations 1, 2 and 4) or improved (observations 3 and 5). Of 4 relapsing patients (observations 6–9), 2 (observations 6 and 7) achieved partial response and a third (observation 9) had clinical improvement and reduction of the bone marrow plasmocytomatous infiltrate from 92 to 64 %. Almost constantly VCR monochemotherapy courses significantly improved the peripheral hematological conditions. The rise of leukocyte and platelet counts in the patients treated with VCR owing to cytopenia deserves special clinical interest, since it allowed the subsequent use of more myelotoxic agents.

The association of VCR to PTC (observations 10–12) has potentiated the effectiveness of this alkylating agent. The total dose of PTC used for inducing remission was greatly reduced (200–320 mg/course) as compared to that administered in PTC monochemotherapy (320–800 mg/course, mean = 600 mg/course) [3]. The treatment times were shortened accordingly. The myelotoxicity of VCR-PTC combination therapy seems somewhat higher than that of PTC alone but we did not observe hematological complications of clinical relevance.

These results demonstrate that VCR is effective against the myelomatous plasma cells. VCR monochemotherapy can be employed not only to stabilize alkylating-induced remissions but also to treat relapsing

myelomas with complicating cytopenia and/or alkylating resistance. This drug is in fact well tolerated and appears to be lacking cross resistance with alkylating agents The rationale for associating VCR *to PTC* is shown by the presented preliminary results obtained in advanced myelomas

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A. Riccardi, G Merlini and C. M. Montecucco, Istituto di Patologia Medica I dell'Università di Pavia, Pavia (Italy)

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Remission of Acquired Factor VIII Inhibitor Induced Bleeding

To the Editor

Acquired factor VIII inhibitors and bleeding have been described in patients with collagen disorders, ulcerative colitis, after normal delivery monoclonal gammopathy drug-induced disorders and in apparently normal people without a detectable cause [1]

A patient with acquired factor VIII inhibitor presenting with haemophilia-like bleeding in joints and soft tissue in whom complete remission was obtained after treatment with factor VIII inhibitor bypassing activity (Feiba), prednisolone and cyclophosphamide is decribed. Her factor VIII level remained normal and factor VIII inhibitor undetectable.

Case Report

A 68-year-old female was admitted to hospital in January 1978 with severe bleeding in her arms, knee joints and legs; there was history of recurrent bruises for the last 2 years. Family history of bleeding disorders was negative. Haemoglobin 9 g/dl platelets: 295 × 10⁹/litre; bleeding time prothrombin time, fibrinogen and liver function test were normal. Partial thromboplastin time: 96 sec two-stage factor VIII assay: 2.7% factor VIII-related antigen: 192% (Laurell technique) and factor VIII inhibitor 8.5 U/ml. She was treated with blood transfusion, human plasma fraction Feiba, prednisolone and cyclophosphamide. Her bleeding stopped rapidly and her factor VIII rose to 59% and factor VIII inhibitor fell gradually to undetectable levels. There was no evidence of disseminated intravascular coagulation and her platelet counts remained normal during and after Feiba infusion. Cyclophosphamide and prednisolone were eventually stopped in June 1979 without further bleeding, factor VIII was 118% and factor VIII inhibitor remained undetectable.

Discussion

It is extremely difficult to achieve haemostasis in patients with factor VIII inhibitor especially with high titres. Large doses of factor VIII infusion, plasmapheresis have been used in these cases. *Preston et al.* [3] have found Feiba to be effective in controlling external bleeding in patients with factor VIII inhibitor but others found no real benefit. Steroids with or without immunosuppressives have been used with some effect in patients suffering from collagen disorders associated with inhibitors. In patients with low-titre inhibitors, *Nilsson et al.* [2] were able to suppress factor VIII inhibitor with cyclophosphamide. In this patient, rapid

myelomas with complicating cytopenia and/ or alkylating resistance This drug is in fact well tolerated and appears to be lacking cross resistance with alkylating agents. The rationale for associating VCR to PTC is shown by the presented preliminary results obtained in advanced myelomas.

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A. Riccardi, G Merlini and C. M. Montecucco, Istituto di Patologia Medica I dell'Università di Pavia, Pavia (Italy)

Original Papers

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Clinical Differential Diagnosis of Hairy-Cell Leukaemia

J Jansen, G J den Ottolander J te Velde and P Lopes Cardozo

J A. Cohen Institute for Radiopathology and Radiation Protection, and Departments of Haematology and Pathology Leiden University Medical Centre, Leiden

Key Words. Bone marrow biopsy Differential diagnosis Hairy-cell leukaemia Monocytopenia Pancytopenia Splenomegaly

Abstract. The data on hairy-cell leukaemia (HCL) and resembling disorders in the literature and in our patients were analyzed to determine which clinical features and laboratory data are important for the recognition of HCL in an early stage.

In pancytopenic patients the typical pattern of bone marrow involvement in HCL and the low number of monocytes in the peripheral blood appear to be essential for the differential diagnosis. In patients with many neoplastic cells in the peripheral blood, the presence of neutropenia and monocytopenia as well as tartrate-resistant acid phosphatase activity in the neoplastic cells, appears to be crucial for early diagnosis. Thus, the clinical features and routine laboratory data alone are sufficient in the majority of cases to suggest the diagnosis HCL. The monocytopenia proved to be most helpful in this respect. Nevertheless, in all patients, and certainly in patients presenting with atypical features, a bone marrow biopsy is indispensable for the correct diagnosis.

Introduction

Hairy-cell leukaemia (HCL, leukaemic reticuloendotheliosis) was first recognized as a distinct clinicopathological entity by *Bouroncle et al* [3] in 1958. The disease accounts for 2–5% of all cases of leukaemia thus, its incidence must be about 0.2–0.5/per 10^6 Although the origin of the pathological cell is still under discussion, there is general agreement on the clinical picture and the histopathological features [11 19 25 26, 48]

From the literature and our own experience it has become clear that the diagnosis of HCL may be extremely difficult. It should be easy to arrive at when hairy cells (HC) are present in abundance in the peripheral blood, but even then many of these cases have been wrongly diagnosed as chronic lymphocytic leukaemia or monocytic leukaemia. In aleukaemic patients the diagnosis is more difficult, and cases have been erroneously diagnosed as myelofibrosis with myeloid metaplasia, malignant lymphoma (NHL), or aplastic anaemia (AA).

haemostasis was achieved after infusion of Feiba and eventual disappearance of factor VIII inhibitor and rise of factor VIII level were probably due to combined steroid and cyclophosphamide therapy although spontaneous remission of her condition was a possibility

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Dr M. L. Ghosh, MBBS, DTM & H, DCH, FRCPI, FRCPG
Department of Haematology
District General Hospital, Barnsley Yorks (UK)

PCL (=48)	SS (n=84)	AMoL (=51)	CMoL (=67)	MH (=71)	MMM (=227)	AA (=131)
1.5	1 8	0 9	1.5	1 7	1 0	21.0
55	61	20–47	66	32	63	38–55
29–76	3?–81	1–82	44–90	1–78	10–94	2 73
eeks to months	years	eeks	months to years	weeks to months	months to years	weeks to months
90	< 5	25	50	10	15	45–75
70	< 5	50	5–50	80	50	<10
50	10	50	40	50	>95	0
70	40	40	30	40	65	0
30	80	60	10	20–70	15	<10
90	0	85	80	50	70	95
< 5	< 5	15	30	40	15	90
90	70	75	50	15	55	< 5
100	100	100	60	0–75	30	0
10	< 5	50	20	30	5	85
60	10	70	60	20–65	20–40	95
1	60	5	18–36	6	18–60	13–35
30, 40, 54 55	14, 15 21 53	5 29 47	18, 36, 46, 57	10, 23, 31 52	4, 43 51	20, 22, 33

tory findings are important for the early diagnosis of HCL, we analyzed the data on HCL and a number of resembling disorders in the literature and our own files. Special attention was paid to the significance of the severe monocytopenia reported to be associated with HCL [45]

Materials and Methods

The frequency of several clinical features and laboratory data of HCL, chronic lymphocytic leukaemia (CLL), prolymphocytic leukaemia (PL), plasma-cell leukaemia (PCL), Sézary' syndrome (SS), other forms of malignant lymphoma (non-Hodgkin lymphoma; NHL), malignant histiocytosis (MH), acute monocytic leukemia (AMoL), chronic monocytic leukaemia (CMoL), myelofibrosis with myeloid metaplasia (MMM), and aplastic anaemia (AA), were calculated from series reported in the literature. Some of these series derive from studies conducted in the Leiden University Medical Centre [20, 21 25 26]. Particularly the clinical and laboratory findings with relevance for the differential diagnosis between HCL and the other entities were noted.

The morphology and cytochemistry of the various cell types was studied by cytological examina-

Table I. Clinical and laboratory findings in HCL and related disorders as reported in the literature. When the mean values of a parameter in the different series were rather close, a general mean value was calculated; otherwise, the range of the mean values is given

	HCL (n=337)	CLL (n=1094)	PL (n=27)	NHL (n=1,581)
♂ ♀	4.2	1 9	2	1.5
Age Median	52	64	66	50
Range	19–87	31–90	46–77	1 93
Duration of symptoms	weeks to years	months to years	weeks to months	months
Haemorrhagic diathesis, %	30	10	30	< 5
Constitutional symptoms, %	30	50	60	20
Splenomegaly %	80	50	100	5
Hepatomegaly %	35	30	70	10
Lymphadenopathy %	25	80	60	80
Anaemia (Hb < 12 g/dl) %	80	30	90	10
Leukopenia (< 5×10^9/l) %	70	0	0	< 5
Leukocytosis (>10×10^9/l), %	15	> 95	> 95	5
Leukaemic[1] %	90	100	100	5–10
Neutropenia (< 1.5×10^9/l) %	90	10	10	< 5
Thrombocytopenia (<100×10^9/l) %	75	15	50	10
Median survival[2] months	42	70	12	12–24
References	25 44 48	2, 41 56	1 17 24	42, 49

HCL = Hairy cell leukaemia CLL = chronic lymphocytic leukaemia PL = prolymphocytic leukaemia NHL = *malignant lymphoma (non-Hodgkin lymphoma) PCL = plasma cell leukaemia SS = Sézary's syndrome* AMoL = acute monoblastic leukaemia CMoL = chronic monocytic leukaemia MH = malignant histiocytosis MMM = myelofibrosis with myeloid metaplasia AA = aplastic anaemia.

[1] Neoplastic cells or blast cells in peripheral blood.

[2] Not corrected for age

However not all cells with 'hairy' cytoplasmic protrusions are HC. Thus, other entities may simulate HCL [16 27 37]

As in most other lymphoproliferative disorders, the diagnosis HCL needs confirmation by histological examination For this purpose a bone marrow biopsy is probably the simplest procedure [7] Other diagnostic findings for instance the presence of tartrate resistant acid phosphatase (TRAP) in the pathological cells or of typical rod-like intracytoplasmic structures (ribosome lamella complexes) although helpful are not conclusive, because both can occasionally occur in other disorders as well

The correct diagnosis is certainly not only a matter of theoretical interest, because HCL requires a different therapeutic approach than the forementioned diseases. In particular chemotherapy may provoke life threatening pancytopenia, whereas withholding of all forms of therapy or splenectomy may lead to prolonged survival It is therefore important that the diagnosis HCL be considered in an early stage.

To determine which clinical and labora















Fig. 2. Example of the variable morphology of HC in the peripheral blood. Eccentric, round nucleus, distinct granules in the cytoplasm b Horseshoe or footpad shape of the nucleus ("Rieder cells"). Oval nucleus, distinct nucleolus d Pseudo-binuclear cell Deep nuclear cleft and cytoplasm slip overlying the nucleus f Heavily clumped chromatin as in CLL.

tion of peripheral blood smears from the files of the Laboratory of Haematomorphology of the Leiden University Medical Centre In addition, the numbers of monocytes in the peripheral blood were determined in 10 patients with HCL, CLL, NHL, MMM, and AA and 9 patients with SS For PL, PCL, and MH, too few patients were available for analysis. Because patients with AMoL and CMoL nearly always have elevated monocyte counts, we also omitted these disorders from this part of the study For each patient, the mean number of monocytes on five consecutive occasions before the start of therapy was calculated from a differential count of 200 leukocytes and the corresponding WBC count. Mean monocyte numbers were compared with Wilcoxon's test.

Results and Discussion

The clinical features and laboratory data of HCL and the other entities are listed in table I and some pertinent differences in the incidence of these features in table II The monocyte counts of the patients studied are shown in figure 1 The patients with HCL had significantly lower monocyte counts than the patients with the other disorders ($p < 0.05$) The monocyte counts of AA patients were significantly higher than those of HCL, but lower than those of the other entities ($p < 0.05$) A general description of the clinical findings in the various disorders follows.

HCL predominantly affects middle-aged males Splenomegaly without lymphadeno-

Table II. Some clinical and laboratory findings with relevance for the differential diagnosis

	≥75% of cases	≤10% of cases
Severe constitutional symptoms	MH	CLL, SS, HCL, AA, MMM, CMoL
Splenomegaly	MMM HCL, PL	AA, NHL, SS
Anaemia	AA, HCL, PL, PCL, AMoL, CMoL	NHL, SS
Leukopenia	AA	CLL, PL, PCL, NHL, SS
Neutropenia	HCL, AA	CLL, PL, PCL, NHL, SS, MMM
Monocytopenia	HCL, AA	AMoL, CMoL, MMM, CLL, PL, PCL, NHL, SS
Thrombocytopenia	AA, HCL	NHL, SS

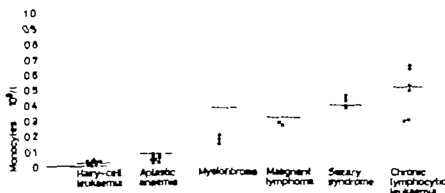


Fig. 1. Monocyte counts of patients with HCL and resembling disorders. Dotted lines denote mean values. The mo ocyte counts of HCL ar significantly lower than those of the other entities ($p < 0.05$; Wilcoxon test) the monocyte counts of AA are significantly high than those of HCL, but lower than those of th other entities ($p < 0.05$).

are rare. Reticulin fibres are increased in a typical network (fig. 3 5). A bone marrow biopsy is highly preferable to bone marrow aspiration, since the typical reticulin net work is not demonstrable on bone marrow smears and the loose diffuse infiltrate can only be recognized in histology

Additional important laboratory findings in HCL are normal or only slightly elevated serum LDH levels and sometimes elevated serum alkaline phosphatase levels. Lysozyme levels are normal or decreased [8] The level of γ-globulin is normal or elevated, hypogammaglobulinaemia is rarely observed. Only one well-documented case of paraproteinaemia has been reported [12]

Pancytopenia is the most frequent presentation in HCL. Some of these patients are easily misdiagnosed as suffering from AA, particularly when splenomegaly is absent. However in AA the duration of symptoms is often shorter haemorrhagic diathesis more prominent, and thrombocytopenia often much more severe. Monocytopenia may be severe in APA (fig. 1) [22] but is less severe than in most cases of HCL (fig. 1). Examination of a buffy coat preparation of peripheral blood is very helpful in these situations, because in cases of HCL it will often show some HC. However histological examination of a bone marrow biopsy specimen is indispensable. In AA it will show – in addition to decreased haematopoiesis – focal or diffuse infiltration of lymphocytes, plasma cells, macrophages, and mast cells [50] In patients with splenomegaly one should hardly ever accept the diagnosis AA.

Patients presenting with splenomegaly as the only abnormal clinical finding often are a diagnostic problem. The various causes of splenomegaly are covered by every textbook of medicine. Again, a thorough examination of a buffy coat specimen for HC and a bone marrow biopsy should lead to the correct diagnosis. In HCL the bone marrow is invariably involved and the pattern of infiltration is very typical. Particularly in patients with

PL	NHL	PCL	SS	AMoL	CMoL	MH
0	0	0	0	± – +	± – +	0 – +
0 – + +	0 – + +	0 – + +	0 – ±	0 – + +	0 – + +	0 – ±
0 – +	0 – +	0 – ±	+	+ – + +	+ – + + +	± – + + +
0 – + + 0 – +	0 – + 0 – ±	0 – + + + 0	+ + 0	0 – + + 0	+ – + + 0	+ – + + + 0 – ±

pathy is usually observed at presentation but in 20% the spleen is not palpable [19 25 44 48] An occasional patient presented with massive lymphadenopathy [6] Only few patients have severe constitutional symptoms at presentation although many complain of repeated recent infections in particular of the upper and lower air passages. Easy bruising is sometimes observed, but severe haemorrhagic diathesis is rare Most of the patients are pancytopenic: in particular neutropenia (90%) and monocytopenia (90%) are very pronounced. Only 15% of the patients show mild leukocytosis at diagnosis and WBC counts of over 50 × 10^9/l are rare (± 1%) When leukocytosis is present, many HC are found in a peripheral blood film, but in leukopenic cases HC are sometimes completely absent from the peripheral blood or are only found after thorough examination of a buffy coat preparation HC are large usually measuring 10–18 μm. The excentric nuclei show a markedly variable configuration with a round, ovoid, bean-shaped dumbbell horseshoe or cloverleaf shape (fig. 2) The nuclear membrane is distinct, the chromatin pattern delicate lacy rather than clumped (as seen in mature lymphocytes) but less so than in blast cells. Small nucleoli are sometimes present, and occasionally are prominent. The large amounts of pale, greyish-blue cytoplasm have a delicately mottled appearance sometimes small azurophilic granules or small vacuoles are seen.

The fine irregular filamentous cytoplasmic projections from which the HC obtained its name are typical The cytochemical pattern of HC is shown in table III TRAP is present in about 90% of the cases and between 2 and 90% of all HC. Cases without TRAP should be thoroughly reinvestigated before they are accepted as HCL.

On the other hand, the presence of TRAP alone does not mean that the diagnosis HCL is certain. The red cell series in HCL usually shows anisocytosis, poikilocytosis, and macrocytosis in most of the patients we have studied, at least some peripheral normoblasts were present. The neutrophil series shows a moderate shift to the left up to the myelocyte, and often toxic granules are present. The neutrophil alkaline phosphatase (NAP) index is mostly markedly elevated [9 25] The platelets, although functionally often abnormal [32] do not show morphological abnormalities. Bone marrow can be aspirated in only half the cases of HCL. If fragments are obtained, they show variable degrees of cellularity with a diffuse infiltration of HC, which account for 20–90% of the nucleated cells. The bone marrow biopsy specimen shows a typical loose infiltration with HC intermingled with erythroid cells and scattered plasma cells (fig. 3). Marrow involvement in HCL is either partial or complete but never nodular Mitoses

Table III. Cytochemical patterns of the neoplastic cells of HCL and resembling disorders

	HCL	CLL
Peroxidase (or Sudan black B)	0	0
Periodic acid-Schiff (PAS)	0 – +	+ – ++
Non-specific esterase	0 – +²	0 – ±
Acid phosphatase		
– tartrate	+ – +++	0 – ±
+ tartrate	0 – ++	0

¹ Sometimes intranuclearly positive.
² Small granules.
³ Paranuclearly positive.
tartrate = after preincubation with tartaric acid

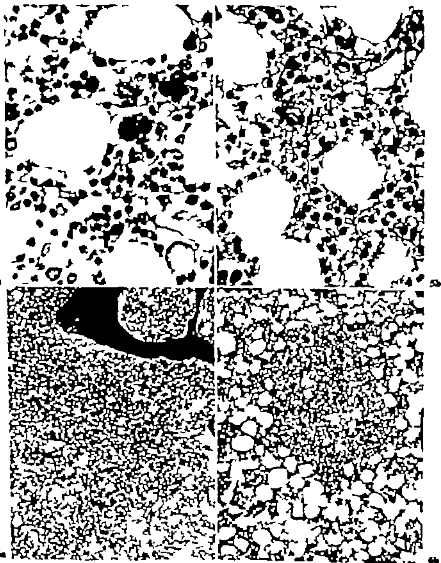


Fig. 5. Incipient infiltration of HC (methacrylate embedded). Only few HC are intermingled with normal megakaryocytes and nests of erythropoesis. Giemsa. × 600. b The typical reticulin network is already present. Gomori. × 600.

Fig. 6. Pattern of infiltration. Diffuse loose infiltration of HCL. b Nodular compact infiltration of malignant lymphoma. Methacrylate embedded Giemsa. × 100.

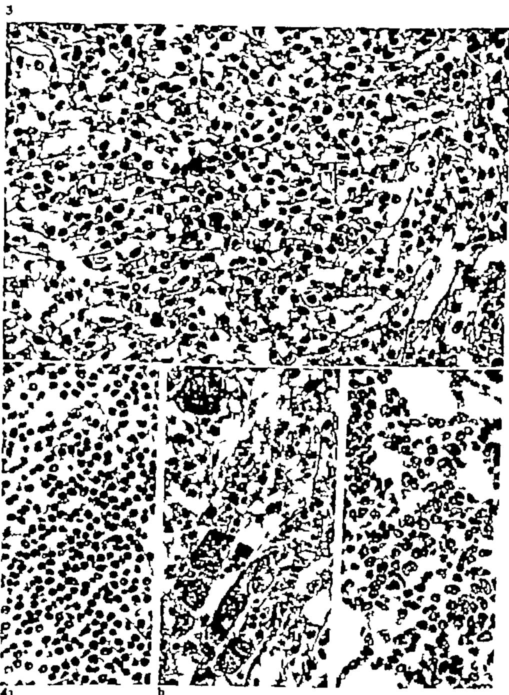


Fig. 3. HCL, bone marrow biopsy specimen showing the fine reticulin network and a loose infiltration of HC. Methacrylate embedded. Gomori × 400.

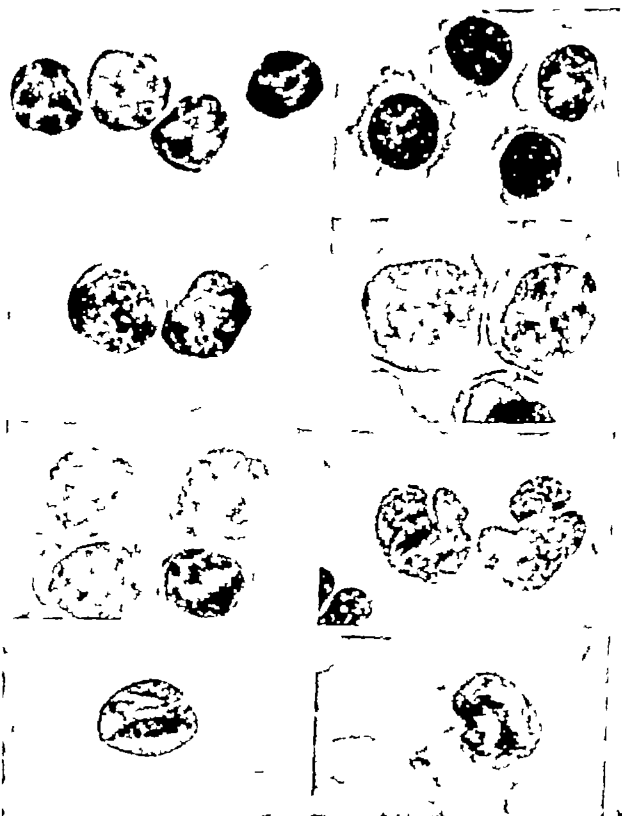
Fig. 4. The fibre network of other haematopoietic malignant processes. a CLL: note the compact infiltration of small cells and the almost complete absence of fibres. b Myelofibrosis: prominent proliferation of megakaryocytes and collagenous bundles. c AMol: irregular proliferation of polymorphous cells, no increase of fibres. Methacrylate embedded, Gomori × 400.

severe splenomegaly dry taps on bone marrow aspiration, and a leukoerythroblastic peripheral blood film, the diagnosis myelofibrosis (MMM) is often made. In MMM, however pancytopenia is rare, severe monocytopenia does not occur and the red cell picture is much more pathological. The pattern of marrow fibrosis in MMM with collagenous bundles is essentially different from the network of reticulin fibres in HCL (fig. 4b). Patients with many HC in the peripheral blood may be diagnosed as having CLL, PL, or some other lymphoproliferative disorder or a monocytic malignancy

CLL occurs at a somewhat older age than HCL. Lymphadenopathy is usually more prominent than splenomegaly but 4–6% of the patients present with modest splenomegaly without lymphadenopathy [13] CLL is not associated with leukopenia. Furthermore, in early cases anaemia, thrombocytopenia, and in particular neutropenia and monocytopenia are rarely seen. Unlike the situation in HCL, the γ-globulin level is decreased in most of the patients In 5% of the cases a paraprotein is present. The pathological cells of CLL are smaller (6–12 μm) than the HC, show a centrally located round nucleus with a heavily clumped chromatin (mosaic-like pattern, fig. 7a) The scanty cytoplasm, forming only a thin rim around the nucleus, has a sharp outline, although sometimes pikes may be present. Often many smudge cells are found. The CLL cells are sometimes weakly and unipolarly positive for acid phosphatase but for TRAP the reaction is negative. The pattern of bone marrow infiltration differs considerably from that of HCL by its pattern of fibrosis. When the bone marrow is extensively involved, the CLL cells lie in compact cellular fields (fig. 4a). In PL [17] the neoplastic cells may occasionally show TRAP activity [34] However hyperleukocytosis with WBC counts of 100–600 × 10^9/l is the rule neutropenia and monocytopenia are usually absent. The malignant cells of PL have round nuclei with one prominent central nucleolus and a distinct cytoplasmic border (fig. 7e) SS is invariably accompanied by erythroderma and palmar and plantar hyperkeratosis. Pancytopenia and splenomegaly are usually not present at the time of diagnosis. Other types of malignant lymphoma (NHL), a term which comprises a large group of different entities and cell types, usually present with either localized or generalized lymphadenopathy Few patients present with pancytopenia [28] and only 5–10% are leukaemic at the time of diagnosis. When involved, the bone marrow often shows on biopsy a patchy or nodular infiltrate comprising the cells of the type of lymphoma concerned (fig. 6b). PCL may present with malignant cells having 'pseudopodia (fig. 7b) and a non-specific esterase pattern identical to that of HC.

HC have some cytological resemblance to cells of monocytic malignancies. The cells of all these malignancies (from acute monoblastic leukaemia [35] to malignant

Fig. 7 Cellular morphology of disorders that must be distinguished from HCL. a CLL heavily clumped chromatin, scarce cytoplasm b PCL round nucleus, perinuclear halo, cytoplasm extension c Leukaemic NHL, only one of the many possible types is illustrated. d AML large undifferentiated cells with abundant cytoplasm showing protrusions e PL centrally located prominent large nucleolus, abundant cytoplasm. f CMoL irregular nuclear contour and abundant cytoplasm with vacuoles g SS cerebriform nucleus. h Malignant histiocytosis neoplastic macrophage with eccentric nucleus, cytoplasm with pseudopodial extensions.



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Multiple Myeloma and Acute Myelomonocytic Leukemia Simultaneous Occurrence without Previous Chemotherapy

Luciana Annino, Pietro Martino, Paola Barsotti, Pietro Serra, Vittorio Marinozzi and Franco Mandelli

Department of Hematology, Department of Internal Medicine and Department of Pathology, University of Rome, Rome, Italy

Key Words. Acute leukemia · Cytogenetics · Electron microscopy · Multiple myeloma

Abstract. A patient with acute myelomonocytic leukemia was found to have IgG para protein on serum electrophoresis, Bence Jones κ proteinuria and increased plasma cells (30%) on marrow examination. The simultaneous occurrence of the two diseases was well documented by cytochemical, immunological and electron-microscopic findings. Bone marrow chromosome investigations showed an abnormal karyotype: hypodiploidy was prevalent and marker chromosomes were present. A possible relationship between acute leukemia and multiple myeloma is discussed.

The possible association in the same patient of multiple myeloma (MM) with other neoplastic diseases such as Hodgkin's disease, chronic lymphocytic leukemia and, above all, acute leukemia (AL) is well known [14]. Numerous cases have been reported in the literature in whom acute nonlymphocytic leukemia (ANLL) complicated and concluded the course of MM. It has been suggested that in such cases a therapy with alkylating agents had a leukemogenic effect [3, 8].

However different problems arise when attempting to explain the rare cases in whom the leukemic process started prior to any chemotherapy 1–3 months after MM diagnosis [9, 14, 15, 17] and the even more exceptional cases of simultaneous onset of MM and ANLL in untreated patients [5, 13, 16].

We report a case in whom MM and myelomonocytic acute leukemia were diagnosed simultaneously and who owing to the cytological, immunological and cytogenetic evidence, allows a thorough discussion of the diagnostic and explanatory aspects.

Case Report

A 74-year-old male suffering from asthenia and dyspnea following physical effort was hospitalized in March 1978. On examination, he presented intense paleness and right lateral pleural effusion of transudate type without pathological cells. There was neither hepatosplenomegaly nor enlarged lymph nodes.

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J Jansen MD Department of Haematology Isolation Ward, Building 16, Leiden University Medical Centre, Leiden (The Netherlands)

plasma cells and monoblasts in the liver sinuses. In the spleen, we found no lymphoid follicles and in the red pulp w noted numerous plasma cells and few monoblast-like blasts similar to those found in the liver The bone marrow was hypocellular ith marked hypoplasia of the normal series; there was still moderat number of blast cells with occasional plasma cells. In the renal cortex there ere lesions typical of 'shock kidney' In the collecting tubules there were numerous eosinophilic cast The tubular epithelium showed some necrosis but there were also some other degeneration marks such as large cells with olumlnous and hyperchromatic nucleus Syncytial lements were absent

Special Studies

Cytogenetic investigations were carried out using direct peripheral blood and bone marrow preparations [1]

Numerical and structural abnormalities were found in 100%/ metaphases. Modal number ranged from 44 to 90 chromosomes, with a prevalence of cells (20 out of 25) at chromosomes 44 and 45 In the hypodiploid metaphases, the loss involved mainly chromosomes 14 16, 18 and 19 On the contrary gain in chromosomes was

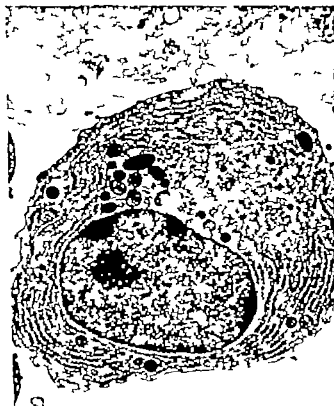


Fig. 3. Bone marrow plasma cell showing moderate nucleo-cytoplasmic asynchrony (not the mainly scattered pattern of chromatin and highly active cytoplasm). F+°r°4 Ur+Pb, × 12,000.

The following laboratory tests were carried out hematocrit 13%, hemoglobin 4 g/dl red blood cells 1,200,000/μl, white blood cells 10,800/μl, with 62% neutrophils, 11% lymphocytes, 1% eosinophils, 8% monocytes, 10% monocytoid blasts, platelet count 20,000/μl, reticulocyte count 6,000/ml ESR (Westergren/1 h) = 145 mm Total serum protein 6 g/dl 62% albumin, 3% α-globulin, 7% α globulin, 12% β-globulin and 16% γ globulin.

The electrophoresis showed a monoclonal spike in the β region. Quantitative immunoglobulin levels showed IgG 1,974 mg/dl IgA 35 mg/dl and IgM 101 mg/dl The serum immunoelectrophoresis showed an increase and a dispersion in the IgG range and a modest diminution of the IgM and IgA Urine: in two tests, Bence Jones proteinuria type K was found. Skeletal X ray examination showed no osteolytic lesions. Bone marrow examination revealed a marked increase of blast cells (45%) with nongranular basophilic cytoplasm and scattered nuclear chromatin with 2–3 prominent nucleoli. There was an increase in plasma cells (30%), some of them were also binucleate and with morular cytoplasms. Erythroblastic and granuloblastic series were hypoplastic and megakaryocytes were absent.

The diagnosis of MM associated with acute myelomonocytic leukemia M4 according to the FAB classification [2] was made on the basis of cytochemical stains such as Perox, PAS and non-specific esterase, using as substrate α-naphthyl butyrate (the latter before and after exposure to NaF fig. 1)

10 days after admission, a lymph-nodal swelling appeared on the back of the patient's neck and a biopsy showed plasma cell infiltration including a number of binucleate cells. The patient was given a 10-day course of melphalan (10 mg/day), prednisone (1 mg/kg/day) and supportive therapy following the treatment, a marrow smear showed a clear reduction of plasma cells (7%) and an increase of blasts (60%).

Soon afterwards the patient worsened and died (May 5 1978) from infection (bronchopneumonia and urinary infection caused by pseudomonas) and hemorrhage.

Autopsy showed cerebromeningeal hemorrhages, bilateral bronchopneumonia, acute lung edema, slight reactive splenitis in the senile spleen and enlarged kidneys. No osteolytic lesions, or lymph node hyperplasia were found. Microscopic examination showed neutrophils and lymphocytes,

1



Fig. 1. Bone marrow cluster of blast cells and plasma cells. May-Grünwald-Giemsa.

2



Fig. 2. Bone marrow: plasma cell reacted with anti-IgG antiserum

trary showed a relative immaturity with one or more large mainly scattered nucleoli and chromatin (fig. 3).

The blast cells were characterized by a high nucleocytoplasmic ratio. Some of them had a large roundish nucleus with scattered chromatin and prominent nucleoli and little cytoplasm containing few organelles. Other blast cells had a larger number of organelles, among which were the Golgi apparatus and some lysosomal granules, these cells were also characterized by roundish or irregularly folded nuclei and cellular profiles indented by numerous digitate cytoplasmic prolongations (fig. 4).

Discussion

The simultaneous diagnosis of MM and myelomonocytic leukemia in our patient was made on the basis of a double population of leukemic cells and myelomatous plasma cells present at onset in the bone marrow

The myelomatous nature of the plasma cell population is evidenced by morphological aberrations, seen both by optical and electronic microscopy characterized by the nucleocytoplasmic maturity asynchronism, a feature of neoplastic plasma cells [3 4] by the positive reaction to immunofluorescence with anti-IgG serum and lastly by the presence of IgG-k monoclonal dysproteinemia with Bence Jones k proteinuria. The leukemic population has the morphological and cytochemical features typical of myelomonocytic forms and does not react with anti-IgG sera. The diagnosis of myeloma is confirmed by the histological results of the lymph node biopsy which showed the presence of a diffuse plasma cell infiltration.

The postmortem findings confirmed the diagnosis, showing a myelomatous infiltration of the liver and spleen and leukemic infiltration of the marrow spleen and liver

Cytogenetic investigation showed numerical and structural alteration in 100% of the mitoses examined in about 50% of the mitoses a marker chromosome was present whereas in 5 hypotetraploid mitoses examined there was a second marker chromosome The alterations in our case are in contrast with the normal findings reported by *Parker* [13] and *Cleary et al* [5] in cases similar to ours on the other hand, they cannot be compared with those found in cases of AL occurring as a second neoplasia in the course of MM insofar as in our case we can exclude the influence of therapy Cytogenetic findings similar to ours have been observed in 50% of patients with ANLL [1] and chromosomal aberrations of various types can also be found in MM [10]

Our case of simultaneous onset of MM and AL shows that the onset of AL in the course of MM is not always a consequence of previous chemotherapy and that other pathogenetic hypotheses must be advanced. The growth of the leukemic clone might be caused or at least favored by a decreased immunological protection caused by myeloma, a disease which has a slow development with a rather long preclinical course [4 13] On the other hand, considering that acute leukemias in the course of MM are almost all of the myelomonocytic type, it could be accepted according to *Osserman's* [12] hypothesis that chronic stimulations of the reticuloendothelial system give rise to plasma cell and monocytic dyscrasia. This hypothesis would seem to find confirmation in the experiments where an intraperitoneal injection of mineral oils can produce both a plasmacytoma and myelomonocytic leukemia in Balb/c mice [18]

found in all groups, mainly in group C. In 13 of 25 metaphases analyzed, a marker chromosome was present deriving from a translocation t(,8) (q ter q13) In the five hypotetraploid metaphases examined a second marker chromosome originating from unidentified chromosomic material on the long arms of chromosome 4 was observed A blurring phenomenon was not found.

Immunofluorescence The bone marrow smears previously fixed in 20‰ methanol were incubated according to *Hijsman et al* [7] with fluorescein-stained human anti Ig antisera. In the slides incubated with anti IgA and anti-IgM antisera there was no evidence of positive plasma cells, while in those incubated with anti IgG antiserum the plasma cells were strongly positive, showing an intense production of IgG (fig. 2).

Electron microscopy The plasma cells were characterized by a moderate nucleocytoplasmic asynchrony the cytoplasm, in fact showed clear evidence of a strong synthetic activity with numerous RER cisternae filled by moderately electrondense material and with greatly enlarged Golgi apparatus tending to occupy a wide cytoplasmic area near the nucleus the nucleus, on the con-



Fig. 4 Bone marrow aspirate: blast cell characterized by a very large nucleolus and digitate cytoplasmic projections: note also the Golgi complex and lysosome-type granules. F+%4 Ur+Pb. × 16,500.

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Hemoglobin E-Thalassemia A Study of 16 Cases

B C Mehta M B Agarwal D G Varandani R H Joshi and A B Bhargava
Dr Jamnadas C. Patel Hematology Department, K E.M. Hospital, Bombay

Key Words. Beta -thalassemia Hb E-delta-beta thalassemia Hb E-thalassemia

Abstract. 16 cases of hemoglobin E-thalassemia are reported. The interaction of thalassemia with Hb E produced a varying severity of E-thalassemia. 10 cases had a thalassemia intermedia type of clinical picture while 6 had a more severe disorder requiring regular transfusions from early childhood. 14 patients were producing Hb-A and therefore had beta type thalassemia whereas 1 case had no Hb-A because of beta type thalassemia. This is in contrast to a much higher frequency of beta thalassemia in cases reported from South-East Asia. Family studies in 1 patient revealed the interaction with delta-beta thalassemia, a situation which is rare. Mating of an Hb E-thalassemia subject with heteroyzgous beta thalassemia resulted in an offspring with severe homozygous thalassemia.

Hb E-thalassemia is a common disorder in South-East Asia. A high incidence is reported in Burmese [5] Thais [8, 10] and North-Eastern Malasians [4] The majority of cases reported from India are from eastern states like Assam and Bengal [2] In the majority of cases reported, interacting thalassemia has been beta type. We report here 16 cases of E-thalassemia, interacting thalassemia being of beta type in the majority and delta-beta type in 1 case.

Material and Methods

Standard hematological techniques as described by *Dacie and Lewis* [3] are used. The

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presence of Hb-A was considered t indicate the beta type of interacting thalassemia whereas the absence of Hb-A indicated interaction with beta type thalassemia. If the heterozygous parent or sibling showed raised Hb F (> 5%), normal or reduced Hb A_2 and heterogenous distribution of Hb F in red cells, the interacting thalassemia was of delta-beta type.

Hematological data of the 16 cases of Hb E-thalassemia is shown in table I and figure 1 Five families were from Maharashtra (Western India); four belonged to backward communities (Mahar 3, Agri 1), while one was Maratha. Two families hailed from Gujarat (Western India): one was Hindu and one Muslim Two families originated from Uttar Pradesh (North India): one was Hindu while another was from backward community (Kori). One was Christian from Tamil Nadu (South India) while there was one case each from Assam and Bengal (Eastern India), areas know for high prevalence of Hb E.

Finally one could also hypothesize, even bearing in mind the fact that MM and AL are different clinical entities, that there are transitory forms already present at onset of the disease, but only rarely demonstrable, as in our case in whom the therapy by interrupting the balance between the two clones, would facilitate the emergence of the more aggressive leukemic form.

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Dr Luciana Annino,
Department of Haematology
Via Chieti 7 00161 Rome (Italy)

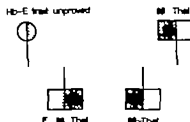


Fig. 2. Family tree of case 15 (HbE-delta-beta thalassemia)

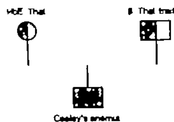


Fig. 3. Family tree of case 8 (HbE-thalassemia having child with Cooley's anemia).

severe cases with low hemoglobin, gross hepatosplenomegaly with poor physical growth and marked skeletal changes [11] and at the other end of the spectrum are mild cases with moderate anemia, splenomegaly and normal physical and sexual development (as in our case 8). The majority of cases have intermediate severity [10] Severity is determined by the type of interacting thalassemia cases with beta thalassemia being more severe than beta thalassemia. Hb A was demonstrated by *Weatherall and Clegg* [11] in 2 Indian brothers in the past. In the present group, 14/16 cases had beta disorder However clinical severity was variable even in these 14 cases, 6 cases needed regular transfusions from early childhood and 8 cases had hemolytic facies. Whereas 10 cases of beta type described by *Wasi et al.* [10] had a mild to moderate disorder none needed regular transfusions and only one had hemolytic facies.

Double heterozygosity for delta-beta thalassemia and an abnormal hemoglobin affecting beta chain is not very common. Of the five families reported [9 10] Hb-S was involved in three, while Hb C and Hb E were involved in one family each [10] The present series includes a family (of case 15) with interaction between delta-beta thalassemia and Hb E. This combination in a case described by *Wasi et al* [10] had produced a mild disorder with an Hb level of 12.7 g/dl and there was no Hb A [10] Whereas our case had a severe disorder presenting with a hemolytic facies, a spleen of 9 cm, Hb of 3.8 g/dl and with Hb-A. Heterozygous delta-beta thalassemia are not uncommon in India and it formed about 15% of all the heterozygous thalassemia seen at our centre [1]

In two reported matings between an individual with Hb E-thalassemia and an individual with thalassemia minor there were no normal offspring out of a total of 16 children [6, 7]. Case 8 in the present series had only one offspring (fathered by a beta thalassemia heterozygote) with severe thalassemia major needing regular blood transfusions.

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The age ranged from 11 to 23 years (mean 11 72 years) 10 patients had one or more episodes of jaundice 8 had hemolytic facies Hepatosplenomegaly was noted in all liver and spleen sizes ranged from 2–12 and 3–15 cm below the costal margin, respectively Hemoglobin range was 2.4–9.2 g/dl. Reticulocyte ranged from 0.8 to 12.0% Red cell osmotic fragility at 0 4% buffered saline varied from 23 6 to 66.3% (mean 42.6%) Hb E varied from 22.9 to 86.5% while Hb F varied from 9.0 to 36.6% Hb A was present in all except perhaps 1 case (No. 11 in table I) One parent and a sibling of case 15 (table I) revealed Hb F of 11 4 and 8.6% respectively and decreased Hb A (1 1 and 1.3%). On the basis of this, it was concluded that the interacting thalassemia in case 15 was of delta-beta type (fig. 2). Case 8 had delivered a child with severe thalassemia major (Hb 3.8 g/dl at the age of 3 months) her husband being thalassemia heterozygote (Hb A_2 4.8% and Hb F 2.4%) (fig. 3).

Discussion

Clinical severity of E-thalassemia is variable [11] At one end of the spectrum are

Table I. Clinical and hematological findings in 16 cases of HbE thalassemia from 12 families

Family No.	Case No	Age years	Jaundice	Hemolytic facies	Liver cm	Spleen cm	Hb, g %	Retic, %	Hb-E, %	Hb-F %
1	1	20	–	+	2	15	8.4	5 4	27.8	35.0
2	2	20	+	+	6	10	5 4	4.8	40 0	28 0
	3	18	+	–	3	8	7 4	4.5	48 3	21 0
3	4	13	–	–	3	6	9.2	10.8	45 7	36 6
4	5	16	+	–	4	9	8 4	4 0	59.8	27.0
	6	6	–	–	3	11	6 9	1.8	72.1	15 6
	7	5	–	–	3	11	6 0	0 8	58.0	23 6
5	8	22	–	–	2	12	7 9	2 0	50.3	9 0
6	9	7	–	–	7	15	4 4	3 0	27 6	13.5
	10	15	+	–	3	10	4 8	2 0	22.9	15 1
7	11	23	+	+	12	14	5.5	12.0	86.5	13.5
8	12	7	+	+	3	8	3 2	12.0	78 0	19.2
9	13	7	+	+	5	5	7.6	4 5	66.3	13.8
10	14	4	+	+	2	4	5 0	5 0	37.2	20 0
11	15	2	+	+	5	8	3.8	6 2	50 9	32.8
12	16	1 /	+	+	3	3	2.4	8 0	60 0	15 5

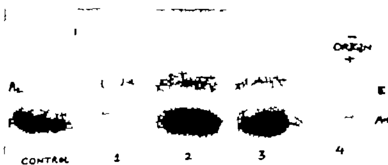


Fig. 1 Paper electrophoresis at pH 8.6. From left to right Control, (1) case 2 (HbE 40 0%) (2) case 14 (HbE 37.2%) (3) case 9 (HbE 27.6%), and (4) case 15 (HbE 50.9%).

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Assessment of Iron Stores in Subjects Heterozygous for β-Thalassaemia Based on Serum Ferritin Levels

J. Economidou, O. Augustaki, V. Georgiopoulou, H. Vrettou, S. Parcha and D. Loucopoulos

Hellenic Red Cross Blood Research Laboratory, Thalassaemia Unit, and 1st Department of Medicine, University of Athens, Athens

Key Words. Thalassaemia · Ferritin · Pregnancy

Abstract. Serum ferritin concentration was assessed in male and female pregnant and non-pregnant thalassaemia carriers and in normal subjects of both sexes. Low ferritin levels were found in 61% of non-pregnant and in 32% of pregnant female β-thalassaemia heterozygotes whereas male thalassaemia carriers had normal iron stores. Increased ferritin levels were not observed in any of the subjects examined. These findings show that iron deficiency is a common finding in female thalassaemia carriers of reproductive age who are not receiving iron supplementation.

In the heterozygous form of β-thalassaemia the haematological abnormality usually seen is hypochromia and microcytosis of the red cells which is sometimes associated with an abnormally high red cell count. Serum iron has been reported as normal or slightly higher than normal [4]. Mild or moderate anaemia is a common finding in such cases and this is largely considered as resistant to iron treatment [13]. Thus, iron supplements are as a rule not given in moderately anaemic patients with heterozygous β-thalassaemia who are generally thought as iron loaded. However, some β-thalassaemia carriers develop severe anaemia during pregnancy which is usually treated with blood transfusion.

We think that it is very important to investigate the iron stores in female subjects with thalassaemia trait. Serum iron concentration is not always a good index of iron stores in these patients. The development of an immunoradiometric assay for measuring circulating ferritin concentrations [1] has made it possible to estimate the amount of storage iron in the body and has proved valuable in investigating patients with iron deficiency or iron overload [9].

In this study we have measured the serum ferritin levels in pregnant women with heterozygous β-thalassaemia and compared them to the findings in other thalassaemia carriers and normal subjects.

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B C Mehta, Dr Jamnadas C. Patel, Hematology Department, K. E. M. Hospital, Bombay 400 012 (India)

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Assessment of Iron Stores in Subjects Heterozygous for β-Thalassaemia Based on Serum Ferritin Levels

J. Economidou, O. Augustaki, V. Georgiopoulou, H. Vrettou, S. Parcha and D. Loucopoulos

Hellenic Red Cross Blood Research Laboratory, Thalassaemia Unit, and 1st Department of Medicine, University of Athens, Athens

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Abstract. Serum ferritin concentration was assessed in male and female pregnant and non-pregnant thalassaemia carriers and in normal subjects of both sexes. Low ferritin levels were found in 61 of non-pregnant and in 32% of pregnant female β-thalassaemia heterozygotes whereas male thalassaemia carriers had normal iron stores. Increased ferritin levels were not observed in any of the subjects examined. These findings show that iron deficiency is a common finding in female thalassaemia carriers of reproductive age who are not receiving iron supplementation.

In the heterozygous form of β-thalassaemia the haematological abnormality usually seen is hypochromia and microcytosis of the red cells which is sometimes associated with an abnormally high red cell count. Serum iron has been reported as normal or slightly higher than normal [4]. Mild or moderate anaemia is a common finding in such cases and this is largely considered as resistant to iron treatment [13]. Thus, iron supplements are as a rule not given in moderately anaemic patients with heterozygous β-thalassaemia who are generally thought as iron loaded. However, some β-thalassaemia carriers develop severe anaemia during pregnancy which is usually treated with blood transfusion.

We think that it is very important to investigate the iron stores in female subjects with thalassaemia trait. Serum iron concentration is not always a good index of iron stores in these patients. The development of an immunoradiometric assay for measuring circulating ferritin concentrations [1] has made it possible to estimate the amount of storage iron in the body and has proved valuable in investigating patients with iron deficiency or iron overload [9].

In this study we have measured the serum ferritin levels in pregnant women with heterozygous β-thalassaemia and compared them to the findings in other thalassaemia carriers and normal subjects.

Material and Methods

52 pregnant women with heterozygous β-thalassaemia aged 18–37 years who were undergoing prenatal diagnosis for thalassaemia were included in this study. These women were approximately in their 16th week of gestation and were not taking any iron therapy. As control were examined 18 female and 16 male healthy thalassaemia carriers who were parents of children with β-thalassaemia major and 49 healthy subjects (24 males and 25 females) in reproductive age. The laboratory investigations comprised the following tests: haemoglobin and serum iron were estimated using standard techniques. For the screening for thalassaemia trait Hb electrophoresis on cellulose acetate using a discontinuous buffer system [8], estimation of HbA_2 by DEAE column chromatography [2] and of alkali resistant (Hb F) by the method of *Singer et al.* [11] were carried out. Serum ferritin concentration was measured by radioimmunoassay using commercially available reagents (Ramco Lab. Inc., Houston, Tex).

Results

The mean serum ferritin levels and the values of the other haematological parameters in the female and male subjects tested are shown in tables I and II. Among the healthy controls, as expected, the serum ferritin, blood Hb and serum iron levels were lower in the female than in the male subjects and the difference was statistically significant with values of $p < 0.001$, $p < 0.001$ and $p < 0.01$, respectively. For the healthy females there was a positive correlation between serum ferritin and iron levels ($r = 0.74$, $p < 0.001$) but this was not observed in the healthy males ($r = 0.14$, $p > 0.5$).

Iron stores were generally low in the healthy women as 14/25 (56%) had ferritin levels below 20 μg/l and 8/25 (32%) had

Table I. Mean values of serum ferritin, blood haemoglobin and serum iron in female healthy subjects and thalassaemia carriers

Subjects examined	Cases	Serum ferritin μg/l	Blood Hb g/dl	Serum iron μg/dl
Healthy subjects	25	20±15	13.1±0.8	79±38
Thalassaemia carriers	18	21±21	11.2±1.4	82±43
		p>0.9	p<0.001	p>0.7
Pregnant thalassaemia carriers	52	38±31	10.3±1.3	119±37
		p<0.001	p<0.001	p<0.001

Table II. Mean values of serum ferritin, blood haemoglobin and serum iron in male healthy subjects and thalassaemia carriers

Subjects examined	Cases	Serum ferritin μg/l	Blood Hb g/dl	Serum iron μg/dl
Healthy subjects	24	68±44	14.6±1.2	110±31
Thalassaemia carriers	16	63±45	13.4±1.2	123±38
		p>0.7	p<0.01	p<0.3

levels below 10 μg/l. None of the healthy males had ferritin levels lower than 20 μg/l.

The group of the female non-pregnant thalassaemia carriers had mean serum ferritin values similar to those of the healthy women. Serum iron values also did not differ whereas mean haemoglobin levels were significantly lower in the thalassaemic carriers. It is interesting that 11/18 subjects (61%) had ferritin levels below 20 μg/l and 5/18 (28%) had levels below 10 μg/l. Thus, approximately the same percentage of iron-deficient subjects was observed in the group of healthy women and female thalassaemia carriers. In this case also a weak positive correlation was found between serum ferritin and serum iron concentration ($r = 0.58$, $p < 0.05$)

Pregnant thalassaemia carriers had higher mean ferritin and serum iron levels than both the healthy women and the non-pregnant thalassaemic heterozygotes although the level of significance was lower for ferritin and for serum iron levels. The mean haemoglobin level was, however significantly lower in the pregnant women compared to that of the healthy women or of the female thalassaemic heterozygotes. Serum ferritin levels were below 20 μg/l in 17/52 and below 10 μg/l in 8/52 cases. A positive correlation was observed in the pregnant thalassaemic carriers between serum ferritin and blood Hb ($r = 0.42$, $p < 0.01$) or serum iron ($r = 0.60$, $p < 0.01$)

On comparing the levels of these three haematological values between healthy males and male β-thalassaemia heterozygotes it was found that there was no statistical difference in the mean levels of serum ferritin or serum iron whereas blood Hb was significantly lower in the thalassaemia carriers. Male β-thalassaemia carriers had significantly higher mean serum ferritin, blood Hb and serum iron levels that female carriers.

Discussion

The iron status of subjects heterozygous for β-thalassaemia (thalassaemia minor) has been investigated in detail in some studies. The study of iron absorption has given variable results. *Bannerman* [3] has reported normal values utilising food iron and [59]Fe-labelled inorganic iron, whereas *Crosby and Conrad* [5] have found increased iron absorption using whole-body counting. However this was not associated with increased accumulation of iron and the authors suggest that this might be due to slightly increased iron excretion.

It has already been reported that thalassaemia minor may be complicated by iron deficiency particularly during pregnancy [12]. *Flemming and Lynch* [7] state that in a study of 15 pregnant women with thalassaemia minor 4 had reduced iron stores, 3 had normal stores and 5 had evidence of iron overload.

The present findings based on the estimation of serum ferritin levels confirm that the male thalassaemia carriers are not iron loaded, since their ferritin levels fall within the normal range for healthy male subjects. On the other hand, the female carriers are often iron deficient with depleted iron stores. In this study the non-pregnant β-thalassaemia heterozygotes had a higher incidence of iron deficiency than the pregnant ones. This could be explained by the fact that the non-pregnant females were older (mean age 39 years) than the pregnant ones (mean age 28 years) and had a history of more than one pregnancy. On the other hand, the pregnant females who were un-

dergoing prenatal diagnosis for thalassaemia were having their first pregnancy and were tested early within the first 16 weeks. No control group of normal healthy pregnant women has been included in this study since in their majority are taking as a routine iron prophylactically

Kelly et al [10] have assessed the iron stores in normal pregnancy and have found that ferritin concentration falls progressively in late pregnancy to a low level despite iron supplementation. This implies that average iron stores in women are inadequate to meet the demands of pregnancy These observations and our own findings suggest that iron deficiency is a frequent finding in female thalassaemia carriers of reproductive age and adequate iron supplementation should be given to these subjects after assessment of iron stores by ferritin measurements This is especially important in countries like Greece where iron deficiency is very common among healthy females of reproductive age as shown, in this study from the ferritin levels which are much lower than those reported in England [6 9]

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Dr J Economidou,
Department of Immunology
Evangelismos Hospital, 45 Ipsilantou Street,
Athens 140 (Greece)

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Skin Window Cellularity and Macrophage Changes in Hodgkin's and Non-Hodgkin's Lymphomas

R. J. Sokol, T. E. Durrant and G. Hudson

University Department of Haematology, Royal Hallamshire Hospital, Sheffield, England

Key Words. Lymphoma · Macrophages · Microscopy, electron, scanning · Skin window technique

Abstract. The cutaneous inflammatory response in patients with Hodgkin's and non-Hodgkin's lymphomas was investigated by light and scanning electron microscopy and compared with that of healthy subjects at 24 and 48 h. Both groups of patients showed a reduction in overall cellularity and statistically significant differences in macrophage surface morphology. In macrophages from healthy subjects, microvilli predominated but in lymphoma patients, cells with ruffles and/or ridges (resembling blood monocytes) were more frequent. In healthy subjects, the percentage of macrophages with predominant microvilli increased between day 1 and day 2. It is suggested that both the cellular response and the maturation (or activation) of macrophages are impaired in lymphomas.

In previous investigations, the value of studying the cellular inflammatory response with both scanning electron microscopy [1–3] and light microscopy [4] has been demonstrated. In the present study both techniques have been used to investigate the response in patients with Hodgkin's and non-Hodgkin's lymphomas, with particular reference to the surface appearances of the cells and the overall cellularity of the exudate.

Materials and Methods

Skin window preparations were made on the anterior surface of the forearm. After cleaning with soap and 70% alcohol or chlorhexidine in spirit, the epidermis was scraped away over an area of a few square millimetres with a sterile scalpel blade until exudation (but not bleeding) was noted. The abrasion was wiped with gauze and a sterile coverslip applied. A haemocytometer coverslip was used for light microscopy and a circular one of 13 mm diameter for scanning electron microscopy (SEM). A cardboard square was put over the coverslip and secured in place with surgical tape. Preparations were performed in duplicate or triplicate. At 24 h, the coverslips were removed for examination (day 1 specimens) and replaced by fresh ones which were removed in turn after a further 24 h (day 2 specimens).

For SEM, the specimens were prepared by immersing the coverslips, immediately following removal, in sterile isotonic saline at room temperature. After this initial washing, the specimens were

dergoing prenatal diagnosis for thalassaemia were having their first pregnancy and were tested early within the first 16 weeks No control group of normal healthy pregnant women has been included in this study since in their majority are taking as a routine iron prophylactically

Kelly et al [10] have assessed the iron stores in normal pregnancy and have found that ferritin concentration falls progressively in late pregnancy to a low level despite iron supplementation This implies that average iron stores in women are inadequate to meet the demands of pregnancy These observations and our own findings suggest that iron deficiency is a frequent finding in female thalassaemia carriers of reproductive age and adequate iron supplementation should be given to these subjects after assessment of iron stores by ferritin measurements. This is especially important in countries like Greece where iron deficiency is very common among healthy females of reproductive age as shown, in this study from the ferritin levels which are much lower than those reported in England [6 9]

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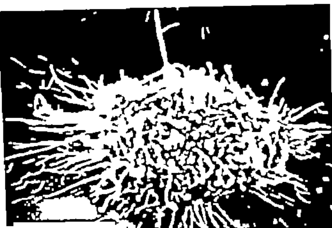


Fig. 1 Scanning electron micrograph from day 2 preparation of normal subject to show macrophage with predominantly microvillous surface appearance. The cell periphery show profusion of filopodia. Marker indicates 5 μm.

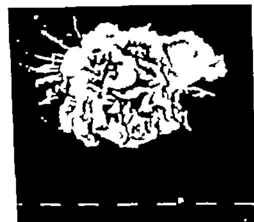


Fig. 2. Scanning lectron micrograph from day 2 preparation from patient with non-Hodgkin lymphoma to show macrophage with predominantly ridged/ruffled surface appearance. The cell periphery shows few filopodia and other processes. Marker indicates 1 μm.

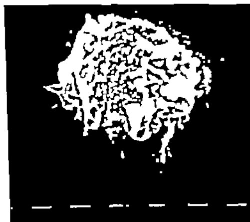


Fig. 3. Scanning lectron micrograph from day 2 preparation from patient with non-Hodgkin lymphoma to show macrophage with mixed surface appearance. Microvilli are the predominant surface feature on the left, while ruffles/ridges predominate on the right. Few filopodia are present. Marker indicates 1 μm.

fixed in cold 1.5% glutaraldehyde in freshly prepared phosphate buffer (pH 7.3) for approximately 6 h (osmolarity 375 mosm/kg) and then washed twice with particle-free sterile distilled water for at least 10 min they were dehydrated through ascending grades of acetone and critical-point dried in a Polaron E 3000 using liquid carbon dioxide. The specimens were sputter coated for 4 min in an Edwards S150 with a gold-plated cathode at 50 mA in an atmosphere of argon and at a constant distance of 30 mm. The preparations were examined in a Philips 501 electron microscope at 15 kV with a viewing angle of 45 and photomicrographs were taken to allow more detailed study The majority of cells could be identified as either granulocytes or macrophages using criteria already described [2, 3] and in some cases their identity was confirmed by light microscopy after the SEM study was completed [3].

For light microscopy the specimens were prepared by air-drying, fixing and staining with May Grünwald-Giemsa. Cellularity was scored for each specimen (without its identity being known) using a 3-point system, representing a poor (score 1), moderate (score 2) or profuse (score 3) exudate.

The main study was carried out on 20 patients with Hodgkin's disease (aged 15–70 years) and 20 patients with non Hodgkin's lymphoma (aged 27–74 years). Light microscopic examination was carried out on preparations from 29 healthy volunteers, and SEM on 7 their ages ranged from 18 to 50 years. Of the cases of Hodgkin's disease, 3 were lymphocyte-predominant, 5 nodular-sclerosing, 6 mixed-cell and 6 lymphocyte-depleted no patient had received chemotherapy or radiotherapy at the time of examination. Of the patients with non-Hodgkin's lymphoma, 2 received chemotherapy and 3 radiotherapy during the course of the skin window studies. In addition, 2 patients had received radiotherapy 2 weeks previously

Semiquantitative studies were carried out on micrographs of macrophages from each of the above groups at a magnification of approximately 10,000× Each macrophage was classified on surface appearances into one of three categories, predominantly microvillous, predominantly ridged or ruffled, and mixed (fig. 1–3) 'microvilli' were defined as small finger like projections, 'ridges and ruffles representing surface folds [5 6]. The classification was carried out by one observer who was unaware of the identity of the subject from whom the preparation was obtained. Cells were studied from both day 1 and day 2 preparations; from each of the preparations, an average of 30 cells was examined.

Results

Light Microscopy

In both groups of lymphoma patients, the exudate appeared generally less cellular than in normal subjects, and statistically significant differences were shown in an analysis of the scoring (table I)

The distribution of cell types was similar in all groups. At day 1 both neutrophil polymorphs and macrophages were usually plentiful, whereas at day 2 macrophages predominated. Eosinophils, lymphocytes, basophils, multinucleated macrophages and effete cells (mainly granulocytes with pyknotic nuclei) were also encountered. The

Table I. Cellularity of exudate (mean scores ± SD)

	Day 1	Day 2	Days 1 and 2
Normal	2.10 ± 0.59	2.21 ± 0.65	2.15 ± 0.62
Hodgkin's disease	1.70 ± 0.73	1.75 ± 0.72	1.73 ± 0.72
Non-Hodgkin's lymphoma	1.63 ± 0.50*	1.70 ± 0.57	1.67 ± 0.53
All lymphoma	1.67 ± 0.62	1.73 ± 0.64**	1.70 ± 0.63

Asterisks indicate differences from the corresponding normal value at 0.05 ( ) and 0.01 ( ) levels of probability

neutrophil polymorphs frequently showed more segmented nuclei and an apparent lack of stained granules when compared with those in peripheral blood smears. In 1 patient with non-Hodgkin's lymphoma, eosinophil granulocytes were particularly prominent.

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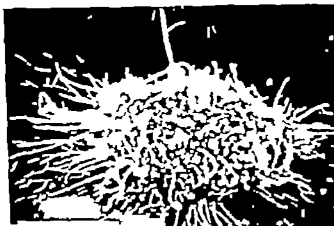


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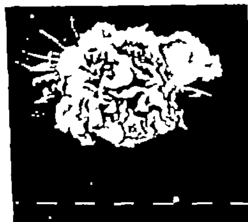


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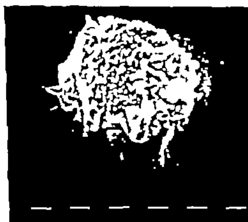


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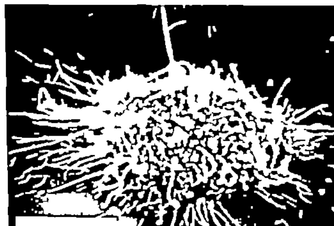


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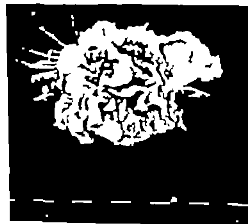


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Fig. 3. Scanning electron micrograph from day 2 preparation from patient with non-Hodgkin lymphoma to show macrophage with mixed surface appearance. Microvilli are the predominant surface feature on the left, while ruffles/ridges predominate on the right. Few filopodia are present. Marker indicates 1 μm.

Table II. Surface appearance of macrophages (% ± SD)

	Day 1	Day 2
Predominantly microvillous		
Normal	74 8 ± 15.2	93.3 ± 5 1[†]
Hodgkin s disease	30 3 ± 20 7	44.9 ± 20.7**
Non-Hodgkin s lymphoma	32.5 ± 21 4	29 4 ± 21.2**
Mixed		
Normal	18.5 ± 11.8	4.2 ± 4 3
Hodgkin s disease	35.6 ± 13 0*	16 6 ± 9.8[††]**
Non-Hodgkin s lymphoma	22.5 ± 11.3	21.6 ± 10 6**
Predominantly ridged/ruffled		
Normal	6.8 ± 3.5	2.5 ± 4.5
Hodgkin s disease	34 0 ± 15 9	38.5 ± 23 6**
Non-Hodgkin s lymphoma	45.0 ± 22.0	49 0 ± 25.0**

Asterisks indicate differences from corresponding normal value at 0 05 ( ) and 0 01 (**) levels of probability Daggers indicate differences from corresponding day 1 value at 0.05 (†) and 0 01 (††) levels.



Fig. 4 Giant form of macrophage from a patient with Hodgkin s disease at day 2. The surface shows a large area of close-packed microvilli with smoother reas to the periphery Numerous long filopodia are present. Marker indicates 10 μm



Fig. 5. Scanning electron micrograph from day 1 preparation of normal subject to show small lymphocyte (bottom left) in close relationship with macrophage. The surface of the lymphocyte is covered with characteristically fine microvilli. Marker indicates 1 μm.

the lymphoma patients, ridged or ruffled forms were often seen (fig. 2). Macrophages having a mixture of microvilli and ridges/ruffles (either in discrete areas or intermingled) were also encountered (fig. 3). The results of the statistical analysis are summarised in table II. Other features of the macrophages included the presence of filopodia (fig 1 4) or broader projections which at the extremes varied from veil like to cord-like. In some of the lymphoma patients, filopodia appeared to be less frequent than normal. Occasionally a giant macrophage was seen (fig. 4).

No differences in surface morphology were observed between granulocytes in the three groups, the fine ridged appearances corresponding to those already described in normal subjects [2] Lymphocytes were occasionally encountered as small round cells showing fine surface microvilli and lying in close contact with macrophages (fig. 5).

Discussion

An important finding of the present investigation is the reduced cellularity of the skin window exudates in both Hodgkin's and non-Hodgkin's lymphomas (table I). Although the earlier literature is somewhat conflicting [7–9] a reduction in cellularity has previously been described in treated cases of Hodgkin's disease [7] In the present investigation, however none of the patients with Hodgkin's disease had received treatment and although 7 out of the 20 patients with non-Hodgkin's lymphoma were treated, a highly significant depression of cellularity could still be observed when only the untreated cases were considered. While the method of assessing cellularity on a three point scale is a relatively inaccurate one, it served to confirm the visual impression and would indicate that the change is disease-related. If one can equate reduced adhesion of cells to the coverslip with reduced migration, the change reflects a defective inflammatory response which could be the result of such factors as humoral inhibition and could be part of the more general impairment of defence mechanisms which is manifested in the increased frequency of infections in malignant lymphoma [10 11]

A striking feature of the macrophages in lymphoma patients was the more variable surface morphology with ridged and ruffled forms being particularly prominent, and conversely microvillous form being less frequent (table II) this confirmed the findings of a smaller preliminary series [3] The ridged/ruffled forms had a resemblance to blood monocytes [6, 12] from which skin window macrophages have been shown to derive [13, 14] The results would therefore seem consistent with a failure of monocytes

to undergo the normal morphological changes of maturation or activation during the inflammatory response. Further evidence for this hypothesis is provided by the observation that in the normal subjects there were significantly more microvillous (and less mixed) forms in the day 2 preparations than at day 1 (table II).

This interpretation is supported by other reports that in malignant disease, monocytes may show a failure to mature: this has been noted *in vitro* with monocytes from patients with malignant melanoma and breast cancer [15, 16]. Also, neoplasms are known to interfere with other normal functions of mononuclear phagocytes [4, 10, 11, 17, 18]. In the light of these earlier observations, the proposed impairment of maturation (or activation) may reflect a general inhibition exerted by malignant tissue on the mononuclear phagocytic system. This may be of considerable importance in relation to the function of macrophages in surveillance against the development of malignant cell lines.

Incidental Findings

Lymphocytes were only occasionally encountered in skin window preparations and on SEM always appeared to be attached to macrophages (*fig. 5*); perhaps they do not gain direct attachment to the coverslip. Their attachment to macrophages may possibly reflect a functional relationship [19]. Lymphoid cells are known to produce soluble substances including, for example, migration inhibition factor, which in addition to immobilising macrophages has been suggested as stimulating their maturation at sites of inflammation [20, 21].

To the best of our knowledge, attention has not previously been drawn to the lack of stained granules in neutrophil polymorphs in skin window preparations. The apparent paucity of granules (which was present in all three groups) presumably reflects loss of stainable material during the inflammatory response. It may be relevant that a marked reduction in alkaline phosphatase activity of skin window neutrophils has been reported by *Janse* [22] and recently confirmed by ourselves in unpublished work, in which the activity of this enzyme in blood and skin window neutrophils was compared in the same subjects.

The surface morphology of giant forms of macrophages has not previously been described in skin window preparations (fig. 4), but it appears similar to that of other macrophages as well as that of giant forms in other situations [23].

Acknowledgements

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R. J Sokol,
University Department of Haematology
Royal Hallamshire Hospital,
Sheffield S10 2JF (England)

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A New Trephine for Closed Bone Marrow Biopsy

Karl Landys

Department of Radiotherapy Sahlgrenska sjukhuset, University of Göteborg, Göteborg, Sweden

Key Words. Bone marrow LM trephine biopsy Osteoblastic metastases

Abstract. Our experience with a new trephine, called the LM trephine, for carrying out bone marrow biopsy is reported This trephine is larger and stronger than most previously described instruments. Two main problems in bone marrow biopsy namely compression and difficulty in obtaining specimens from osteoblastic (or sclerotic) metastases are over come by the special design of the LM trephine tip 310 out-patients with different tumour diseases were examined and no complications were observed.

Microscopic examination of the bone marrow constitutes an essential oncological investigation. The demonstration of tumour cells in the bone marrow is regarded as evidence of generalized malignancy with all the prognostic and therapeutic consequences it entails The classical method of investigation comprises aspiration cytology of the sternum, spinous processes of the lumbar and thoracic vertebrae and the iliac crest with fine needles This technique rapidly became a routine procedure in the investigation of plasmocytoma, and made important contributions to diagnoses in oncology and haematology Several studies have shown that this technique may not be satisfactory for the diagnosis of marrow involvement in malignant lymphomas and metastatic malignancies like small-cell carcinoma of the lung, prostatic cancer breast cancer thyroid cancer and hypernephroma. Among the many workers contributing to the technical and diagnostic development of bone marrow biopsy *Dameshek* [2] *Silverman* [10] *Burkhard* [1] *Radner* [8] *Landys and Stenram* [6] *Helleberg-Rasmussen and Søndergaard Petersen* [4] *Stavem* [11] *Miller and Dennis* [7] *Jamshidi et al* [5] and *Fornasier and Vilaghy* [3] deserve special mentioning.

Material and Methods

Between 1973 and 1977 we used Gidlund's instrument for bone marrow biopsies in 800 out-patients with breast cancer malignant lymphomas and other tumours [6, 9] In 1977 a new type of instrument, called the LM-trephine, was intro-

duced. It was developed at our department and is manufact red by and vailable from Aggtrdens Instrumentverkstad, Sahlgrenska sj khuset, Goteborg, Sweden.

The nstrument consists of hand-powered trephine, faceted mandril and specimen extractor (fig 1). The internal diameter of the trephine is 4 mm, the external diameter 5 1 mm and the total length 162 mm (fig 1). The tip of the trephine has ten teeth. Every other tooth is bent inwards 5 which facilitates removal of the marrow specimen.

Biopsy Technique for Specimen from th Posterior Iliac Crest

Normally the patient sit on 40- to 50-cm-high stool and leans forward with his arms on table But the investigation can also be carried out ith the patient lying on his stomach or side

Under local anaesthesia with 8-10 ml of 2% Xylocaine (Astra), 0 5-cm skin incision is made over the posterior l ac crest, about 5 cm from the midline The bone is punctured at the level of the f rst sacral vertebra, about 7 cm below the top of the iliac crest The lamina compacta is perforated ith the faceted mandril inserted in the trephine. The faceted mandril is then removed and the trephine slowly advanced int the bone marrow with lockwise-counterclockwise motions until adequate marrow is obtained. The t ephine is then withdrawn 2 3 mm and rotated, and its tip tilted at slightly different angle, thus cutting the specimen inside the trephine free from the bone marrow outside the trephine I the next phase, the trephine is slowly withdrawn with alternating rotatory motions. The specimen is removed through the opposit end of the trephine as recommended by *Jamshidi et al* [5]. The biopsy can be performed at right angles or oblique t the surface of the body (fig. 2).

I sclerotic bone, bending of the trephine must be avoided. I such cases the biopsy has t be per formed straight through the bone marrow cavity t the opposit lamina compacta in order to obtain an adequate specimen.

The diameter of the specimen is 4 mm. The length may vary between 10 and 60 mm and the weight between 50 and 350 mg (fig 3)

After control of haemostasis in the puncture channel, the incision is closed with single ski suture The biopsies are taken without premedication as an out-patient procedure. Every patient is examined 10 days after the biopsy

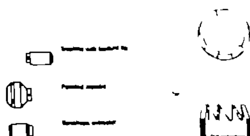


Fig. 1 The LM-trephine

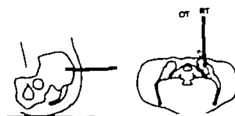


Fig. 2. Biopsy technique from the posterior il iac crest performed at ght angle (RT) or oblique (OT) to the body

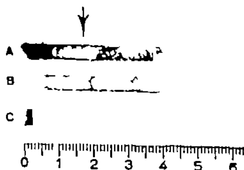


Fig. 3. Specimens of bone marrow obtained with the LM-trephine. A = 38-mm-long sample of inhomogeneous marrow th sclerotic part (arrow). B = 40-mm-long sample of compact sclerotic marrow patient with X-ray-treated metastases of breast cancer in the posterior iliac crest. C = 60-mm-long sample of homogeneous, macroscopically normal red marrow

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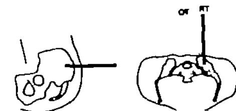


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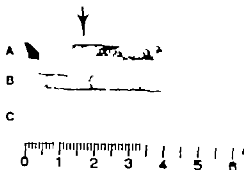


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Fig. 4 *Osteoblastic metastasis* of breast cancer with newly formed woven bone. Focus of *tumour cells around the vessel*. Pronounced fibrosis at top right. Obtained with the LM-trephine. *Haematoxylin and van Gieson.* × 80.

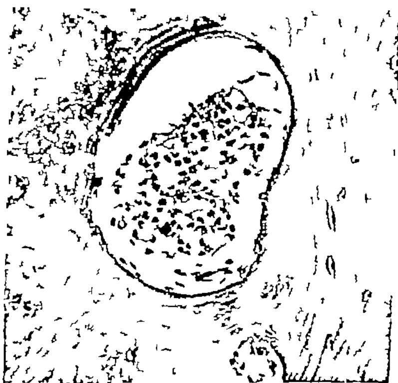


Fig. 5 Osteoblastic metastasis of ductal invasive breast carcinoma. Small focus of tumour seen within dense bony tissue. Obtained with the LM-trephine. Van Gieson. × 200



Fig. 6. Compression of the sample with artefact (crush) on the marrow sample obtained with Gidlund' instrument. HE. × 200.

Results

310 patients underwent bone marrow biopsies with the LM-trephine technique (140 patients with breast cancer 95 with lymphomas, 35 with prostatic cancer and 40 with other tumours). 66 patients complained of tenderness at the site of the trephination for up to 6–12 h after the procedure The pain could be satisfactorily relieved with oral analgesics, e.g. paracetamol. 36 patients had thrombocytopenia, with platelet counts varying between 35 and 97 × 10^9/l. Complications such as bleeding or infection did not occur

Discussion

Closed bone marrow biopsy can be performed with different types of needles or trephines. Each method has its advantages and disadvantages and places certain demands on the patient and on the person carrying out the procedure. The aim of all methods is to obtain sufficient marrow for histological investigation (fig. 4 5). In hyperostosis or pathologically altered bone, the closed biopsy technique may be difficult or impossible. The drill or twist biopsy technique according to Jamshidi or Gidlund, or the punch technique using Radner's or Stavem's needles can only be used when the marrow is of normal density or osteolytic. In sclerotic bone marrow or normal compact bone tissue, the instruments have a tendency to bend and the sample may be damaged by compression (fig. 6). This is well known for sclerotic osseous metastases of prostatic cancer but can even happen in bone-producing osseous metastases of

breast cancer [9] and radiation induced fibrosis in the bone

With the LM-trephine biopsy technique, specimens of good quality could be obtained from all patients investigated, even from those with sclerotic bone metastases. Compression and crushing of specimens could be avoided thanks to the special design of the toothed trephine tip The method ensures the patient minimal trauma, and yields sufficiently large bone and marrow specimens in their intact state as demonstrated in figures 4 and 5 *Histological specimens of this type* and quality are not obtainable by the conventional needle biopsy technique

The LM-trephine is larger stronger and more effective in obtaining biopsies in sclerotic bone and bone marrow than Jamshidi s needle

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Karl Landys, MD
Department of Radiotherapy
University of Goteborg, Sahlgrenska sjukhuset,
S-413 45 Göteborg (Sweden)

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K Cell Activity in Acute Lymphoblastic Leukaemia of Childhood

G Schillirò S Musumeci A Sciotto A Russo and S Marino

Department of Paediatrics, University of Catania, Catania

Key Words. K cell activity Acute lymphoblastic leukaemia Lymphocyte subpopulations Prognosis

Abstract. Lymphocyte subpopulations were studied in 28 children with acute lymphoblastic leukaemia at different stages of the disease. T and B lymphocytes, present in low percentages at diagnosis, increased towards normal during remission in most patients. All patients off therapy had normal values of T and B lymphocytes. K cell activity which was increased during remission in several patients, was normal in all patients off therapy Further prospective studies will establish whether determination of K cell activity is an indicator of the stage of the disease.

Introduction

The identification of T and B cellular surface markers on lymphoblasts is widely used for classification and prognosis of acute lymphoblastic leukaemia (ALL).

The quantitation of the different subpopulations of normal lymphocytes is useful in monitoring the immune status of patients in remission, even if the significance of these data regarding the control of leukaemia and its final outcome is not clear

Patients and Methods

W determined the T B and antibody-dependent cytotoxic lymphocytes (K cells) as per centage of total lymphocyte count in 28 children. These ranged in age from 2 to 12 years and had 'null-cell' ALL at different stages of the disease.

Purified lymphocytes were obtained by frac tionation of heparinized blood on Ficoll-Hypaque density gradient (MSL Eurobio). T lymphocytes were identified by the sheep red cell rosetting technique [1]. B lymphocytes were identified by the presence of fluorescence on their surfaces when treated with anti-whole immunoglobulin conjugate [2; Kappel Laboratories]. K cell activity was determined using ^{51}Cr-labelled chicken red blood cells sensitized with rabbit antiserum [3].

Results and Discussion

At diagnosis, the T lymphocyte percentage was markedly decreased in all patients but reverted to normal during remission in

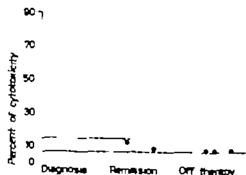


Fig. 1. Antibody mediated cytotoxicity to chicken red blood cells sensitized with rabbit antiserum of lymphocytes from 28 leukaemic children in different stages of disease.

most patients All patients off therapy had normal values. B lymphocytes showed a similar pattern in the three stages of the disease which was characterized by low percentages at diagnosis which increased after complete remission until it reached normal values after cessation of therapy These results are in agreement with the data reported in the literature [4–7] and could be due to the relative increase of circulating normal lymphocytes after remission and to the depressive effects of maintenance chemotherapy on these cells. After cessation of therapy the lymphocytes returned to normal values. K cell activity was above the normal values of our laboratory (10.35 ± 4.34) in 4 of 7 children at diagnosis and in 7 of 15 children in remission (fig. 1) In the latter no correlation was found between K cell activity and clinical factors of risk or time elapsed since remission K cell activity was decreased in 1 and normal in 5 patients off therapy It has been speculated that the K cell is involved in the elimination of tumour cells [8, 9] According to this hypothesis, increased K cell activity during remission should be considered a response to the residual leukaemic cells, while normal activity off therapy should be indicative of minimal or absent antigenic stimuli. On the other hand, the normal or decreased K cell activity observed in some patients during remission could be due either to reduced or absent antigenic stimuli or to immunodepression secondary to therapy Even if the first interpretation seems unlikely because of the lack of inverse correlation with remission duration, the number of cases is too low for definitive conclusions. At present we cannot distinguish between the two possibilities and only further prospective studies will determine whether K cell activity has a prognostic significance If this is the case, serial determinations during remission will provide useful guidelines for the time of stopping therapy and the optimal use of immunotherapy

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Gino Schilirò, MD, Department of Paediatrics, Viale A. Doria, 95125 Catania (Italy)

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Thrombocytosis in Quiescent Chronic Granulocytic Leukaemia after Vincristine and 6-Mercaptopurine Therapy

E. Feliu, F. Cervantes, J. Blade and C. Rozman

Postgraduate School of Haematology 'Farreras Valenti', University of Barcelona, Hospital Clínico y Provincial, Barcelona

Key Words. *Chronic granulocytic leukaemia · Thrombocytosis · Vincristine*

Abstract. In an attempt to retard the appearance of blastic transformation 11 patients recently diagnosed with chronic granulocytic leukaemia were given courses of vincristine and 6-mercaptopurine after stabilization of the disease by busulfan. In 4 of the patients a marked thrombocytosis developed shortly after the administration of such courses. When we compared the clinical and biological features at the moment of diagnosis, the patients in whom thrombocytosis developed after vincristine and 6-mercaptopurine courses showed higher platelet counts and a smaller spleen size than the other ones, although no statistical significance was reached. A possible thrombocytopoietic effect of vincristine is discussed.

Vincristine can be useful in the blastic critical phase of chronic granulocytic leukaemia (CGL) [1, 2]. On the other hand, 6-mercaptopurine (6-MP) is one of the choice agents in the chronic phase of the disease, mainly when thrombocytopenia is present, as well as when blastic transformation develops [3].

Methods and Results

In an attempt to retard the appearance of blastic transformation we started a treatment protocol of patients recently diagnosed with CGL in January 1978. 2 months after a stabilization of the disease was achieved by busulfan, courses of vincristine (0.6 mg/m² i.v., day 1) and 6-MP (300 mg/m² p.o., days 2 and 3) were given every 3 months. Up to date, 11 patients have been included in this protocol. In 4 of them (36.3%), as can be seen in table I, a marked thrombocytosis (mean 1 440 × 10⁹/l) developed after the administration of such courses of chemotherapy, in 3 of them during the first haematological study (1 month after the first course) and in the last patient (case 3) progressively after the third course. 1 patient died from sepsis in the quiescent phase of CGL just before the second course of vincristine–6-MP. The other 3 patients showed persistently raised platelet counts throughout the subsequent courses of vin-

Table I

Patient No	Age years	Sex	Spleen cm	Platelets 10^9 l	Ph chromosome	Platelets 10^9 l at quiescent phase immediately before VCR 6-MP	Platelets 10^9 l after VCR–6-MP zenith
1	49	M		400	–	130	2,600
2*	46	M		1,000		270	1 160
3*	26	F	3	600	–	420	1,000
4	70	F	4	48[illegible]	+	350	1,000
5	17	F	16	700	+	292	275
6	29	F	5	440	+	280	450
7	50	M	4	200	+	190	240
8	45	M	8	130	+	130	220
9	[illegible]3	M	4	150	+	100	140
10	45	M		120	+	100	120
11	54	M	7	400	+	300	374

VCR = Vincristine 6-MP = 6-mercaptopurine
Patients who developed thrombocytosis after chemotherapy

cristine–6-MP The increase in platelet counts was not associated with any significant changes in the remaining haematological parameters. When comparing the clinical and biological features of the patients who developed thrombocytosis with those of the other 7 patients (table 1), no statistical significance was reached, although a high platelet count and a minimal or absent splenomegaly were constant in the patients of the first group when they were first examined. In addition, Philadelphia (Ph) chromosome negativity was only found in patients of this group.

Discussion

Thrombocytosis is very common when CGL first presents itself [4] but is considered as a poor prognostic factor by some authors [4, 5]. On the other hand, slight thrombocytosis after vincristine therapy has been observed in several neoplastic disorders [6] as well as in experiments with laboratory animals [7]. Based on these observations, vincristine has been successfully used in some cases of idiopathic and secondary thrombocytopenia [8]. The mechanism of such thrombocytosis is not completely clear although it could be partially due to a thrombocytopoietic effect [7].

Retrospectively considered, the appearance of thrombocytosis following the administration of a potentially thrombocytopoietic agent in a disorder in which thrombocytopoiesis is generally increased is not surprising [4]. The fact that thrombocytosis developed only in patients with a high platelet count at the moment of diagnosis could support the hypothesis that vincristine acts by potentiation of an increased trend to megakaryocytic differentiation in these patients. Moreover the only mild enlargement

of the spleen in these patients could partially account for the thrombocytosis, if the potential platelet sequestration effect of the spleen is considered. Although striking, the absence of the Ph[1] chromosome only in the patients who developed thrombocytosis does not seem to be related to the latter feature.

As far as we know, the present observation has not been reported to occur in CGL. Its importance comes from the fact that vincristine is being increasingly used in association with other drugs in the chronic phase of CGL. Thus, the appearance of an isolated thrombocytosis in quiescent CGL after vincristine therapy must be due to an effect of the drug rather than to a relapse of the disease. Furthermore, if thrombocytosis is assumed to be a poor prognostic factor in CGL, the question arises, if it is convenient or not to use vincristine in the chronic phase of the disease.

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E. Feliu, Postgraduate School of Haematology 'Farreras Valenti', University of Barcelona, Hospital Clínico y Provincial, Casanova, 143, Barcelona (Spain)

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Multiple Myeloma in Husband and Wife

M. Brugiatelli, M. Comis, P. Iacopino, F. Morabito, F. Nobile, V. Trapani Lombardo and A. Neri

Divisione di Ematologia, Ospedali Riuniti, Reggio Calabria

Key Words. Multiple myeloma · Myeloma cluster · Spouses

Abstract. An instance of multiple myeloma (MM) in a married couple is discussed, since cases of MM in husband and wife have rarely been observed. The patients were aged 60 when the disease was discovered, and the interval between the diagnosis in the mates was 44 months. Genetic or environmental factors that could have explained the occurrence of myelomatosis were not found in these spouses. Therefore, on absence of a defined cause our observation, like others previously reported in the literature, should be considered, although exceptional, a chance event.

Introduction

The occurrence of multiple myeloma (MM) in husband and wife represents a very unusual observation. It was first reported by *Kyle et al.* [5] who published four sets of spouses with MM: the time lag between the diagnosis in the mates varied broadly ranging from 1 month to 15 years. More recently *Pietruszka et al.* [7] described a patient who developed MM 5 years after his wife had died of myelomatosis. All these patients were over 50 when the disease was discovered. No other cases of MM in spouses, to our knowledge, have been so far reported in the literature. We feel, therefore, that it is of interest to present in this communication a new instance of MM in a married couple, who came to our observation.

Case Reports

Husband. A 60-year-old joiner was admitted to our hospital in April 1976 because of a long history of fatigue and lassitude. On admission, renal failure (creatinine 4.6 mg/dl) and severe anemia (Hb 8.3 g/dl) were found. A bone marrow aspirate contained 72% of immature, atypical plasma cells; skeletal survey displayed diffuse osteoporosis. Serum electrophoresis revealed no peaks, but Bence Jones protein, lambda type, was detected by immunoelectrophoresis, consistent with the diagnosis of light chain disease. Chemotherapy with alkylating agents and prednisone was soon after started, and his clinical and hematological conditions rapidly improved.

of the spleen in these patients could partially account for the thrombocytosis, if the potential platelet sequestration effect of the spleen is considered. Although striking, the absence of the Ph[1] chromosome only in the patients who developed thrombocytosis does not seem to be related to the latter feature.

As far as we know, the present observation has not been reported to occur in CGL. Its importance comes from the fact that vincristine is being increasingly used in association with other drugs in the chronic phase of CGL. Thus, the appearance of an isolated thrombocytosis in quiescent CGL after vincristine therapy must be due to an effect of the drug rather than to a relapse of the disease. Furthermore, if thrombocytosis is assumed to be a poor prognostic factor in CGL, the question arises, if it is convenient or not to use vincristine in the chronic phase of the disease.

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familial myeloma have been reported [1 6] But this is not the case with our patients there is no consanguinity their family histories were unremarkable for malignancies, and finally the results of serum investigations in the relati es were negative for immunoproliferative disorders. The role of radiations in determining MM has also been emphasized [3] However we could exclude exposure in both patients. Moreover a careful investigation ruled out the possibility to take into account other environmental factors, such as chemicals or drugs. Finally evidence was achieved that we were not dealing with a community cluster of MM cases [4] they had been married for 20 years, and always lived in the same house this is a single house located on an area where we are not aware of cases of myelomatous recorded in the past years.

In conclusion, all that is lacking in our case reports is the identification of a possible, if any etiological factor(s). In this way the occurrence of MM in these spouses, like in others previously reported in the literature, remains unexplained, and it should be considered, however exceptional, a chance event.

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Alberto Neri, Divisione di Ematologia, Ospedali Riuniti, I-89100 Reggio Calabria (Italy)

Wife. A 60-year-old previously healthy housewife was complaining of weakness and low back pain for several months. When admitted to our department in December 1979 she was anemic (Hb 8.0 g/dl) and a bone marrow aspirate yielded 60% plasma cells, mostly abnormal. Serum protein electrophoresis showed a beta globulin spike, and immunoelectrophoresis disclosed a homogeneous IgA, k, paraprotein. Roentgenograms revealed diffuse osteoporosis and some osteolytic lesions in the skull. The diagnosis of MM, IgA, k was made and a treatment with melphalan and prednisone was administrated.

The main laboratory data of both patients are summarized in table I.

Table I. Main laboratory findings of the patients on their first hospitalization

	Husband	Wife
Hemoglobin, g/dl	8.3	8.0
Leukocytes per µl (× 10³)	5 7	3 6
Platelets per µl (× 10³)	160	150
ESR in 1 h	68	88
Calcium, mg/dl	9 9	9 8
Creatinine, mg/dl	4 6	0.82
Bence Jones proteinuria	yes	yes
Serum immunoglobulins, mg/dl		
IgG	750	410
IgA	38	5 460
IgM	29	10
HLA typing	A1 B5-B17	A9 AW3031 BW21

Family Investigations. The patients do not have children. Sera from two living brothers of the wife and one sister of the husband were examined one brother of the husband could not be investigated. Serum proteins of all these relatives resulted normal when studied by electrophoresis and immunoelectrophoresis.

Table II. The incidence of MM in Reggio Calabria and its surrounding area (420,000 inhabitants) in the last 5 years

Year	Cases of MM
1975	6
1976	7
1977	6
1978	8
1979	7
Total	34

Data have been obtained from all the hospitals of this area.

Discussion

In commenting upon our case reports, the question if MM in this married couple is to be considered as mere chance or if a common etiological factor albeit unknown, might be involved was taken into consideration. The interval of 44 months between the clinical onset of the disease in the husband and the diagnosis in the wife could not be an argument against the latter hypothesis, since it is recognized that the development of an overt myeloma may take a long time [2, 8]

As far as the chance hypothesis is concerned, we estimated that the calculated risk of having MM among the population living in our province is 1 7/100 000/year (table II) then the probability for these spouses to have been afflicted with the same disease is statistically very small.

On the other hand, we were unable to explain the occurrence of MM in this married couple on the ground of a defined cause The etiology of myeloma is largely unknown. Nevertheless, some factors have been claimed. thus genetic factors have been considered, since various instances of

familial myeloma have been reported [1 6] But this is not the case with our patients. there is no consanguinity their family histories were unremarkable for malignancies, and finally the results of serum investigations in the relatives were negative for immunoproliferative disorders. The role of radiations in determining MM has also been emphasized [3] However we could exclude exposure in both patients. Moreover a careful investigation ruled out the possibility to take into account other environmental factors, such as chemicals or drugs. Finally evidence was achieved that we were not dealing with a community cluster of MM cases [4] they had been married for 20 years, and always lived in the same house this is a single house located on an area where we are not aware of cases of myelomatosis recorded in the past years

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Alberto Neri, Divisione di Ematologia, Ospedali Riuniti, I–89100 Reggio Calabria (Italy)

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Blood Group Glycosyltransferase Activities in Plasma from Blood Chimera Subjects[1]

A Yoshida V Davé J Allan and P A Morel

Department of Biochemical Genetics, City of Hope Research Institute, Duarte, and
Los Angeles-Orange Counties Region, American Red Cross Services, Los Angeles, Calif.

Key Words. Blood chimera Blood group glycosyltransferase Galactosyltransferase N-acetylgalactosaminyltransferase

Abstract. Blood group glycosyltransferases (A and B) in plasma are mainly derived from other tissues, not from bone marrow where the blood group substances are synthesized. Therefore, it is possible to determine the original blood type of host subject and grafted cells by examining the enzyme activity in plasma from a chimera subject. Subject WD had 75–85% of O type cells and 15–25% of A_1 type cells A_1 enzyme activity of the subject's plasma was the same as control A_1 plasma, suggesting that subject WD is genetically A_1O or A_1A_1 and O red cells are produced by *OO* bone marrow cells acquired *in utero* Another subject EA has about 85 % of B type and 15% of A B type red cells. B enzyme activity of the subject's plasma is normal but A enzyme activity is only 20% of normal level. Subject EA must be genetically *BO* or *BB* and A gene is restricted to bone marrow cells acquired

Human blood chimerism may originate from vascular anastomosis between dizygotic twins *in utero* or artificially by transplantation of bone marrow cells of different blood types [1] Blood chimera subjects have been occasionally found without twin siblings. This may be a result of one of the twins being adsorbed *in utero* or aborted during its development. The blood group A substance *in red cells* is produced from common H substance by the action of N-acetylgalactosaminyltransferase (A enzyme). and the B substance by the activity of galactosyltransferase (B enzyme) in bone marrow [2, 3] The A enzyme is found in the plasma of blood group A subjects, B enzyme in those who have blood group B and neither enzyme in blood group O individuals. Most of the circulatory plasma enzyme activity is not derived from the bone marrow and only less than one fifth of plasma enzyme is estimated to originate from the bone marrow [4 5] Therefore, it is possible to determine blood types of host subjects and graft

1 This study was supported by Public Health Service Grant HL 20301

ed cells by examining plasma A and B enzyme activities of blood chimera subjects.

We examined the plasma enzyme activities of 2 chimera subjects, i.e. A and O chimera and AB and B chimera. The blood group A and B enzymes were assayed by incubating O red blood cells with the nucleotide sugar (UDP-N-acetylgalactosamine for A enzyme and UDP-galactose for B enzyme) and plasma. The newly produced blood group substance (A or B) on O red cell surface was semiquantitatively assayed using anti-A or anti-B agglutinin with serial dilutions as previously described [6, 7].

Subject WD had 75–85 of O type cells and 15–25 of A_1 type cells, i.e. O and A chimera. The subject has no twin sibling. A_1 enzyme activity of the subject's plasma was the same as control A plasma. No B enzyme activity was detected in the subject's plasma, as was expected from his blood type. O red blood cells obtained from the subject's blood were fully converted to A_1 type after incubation with partially purified A enzyme and UDP-N-acetylgalactosamine, as previously described [6]. Therefore, the subject's O red cells were the same as the common O red cells. These results suggest that the subject WD is genetically A_1O or A_1A and O red cells are produced by OO bone marrow cells acquired *in utero* from the disappeared subject's sibling with O type.

Another subject examined (subject EA) had about 85 of B type red cells and 15 of A_1B type cells, i.e. B and A_1B chimera. The subject has no twin sibling. B enzyme activity of the subject's plasma was the same as that of the control B plasma. However A enzyme activity of the subject's plasma was only about 20% of that of control A_1 plasma. These results suggest that subject EA is genetically BO or BB and A gene is restricted to bone marrow cells acquired *in utero* from the subject's twin sibling with genotype A_1B.

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A. Yoshida,
Department of Biochemical Genetics,
City of Hope Research Institute,
Duarte, CA 91010 (USA)

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Fig. 1. Bone biopsy showing marked marrow necrosis and lack of normal hematopoietic elements. HE. × 100

The neurologic examination revealed right Babinsky sign. No other neurological signs were present. Laboratory investigations showed microscopic hematuria without proteinuria. The ESR (1 h) was 70 mm, hemoglobin 10.2 g/dl, reticulocytes 0.7%, white blood cells 12,200/mm with normal differential count. The peripheral blood smear showed marked anisopoikilocytosis, with normoblasts.

Coagulation studies disclosed the following results: platelets 48,000/mm, bleeding time 3 min, clot retraction 30%, clotting time 8/9 min, prothrombin time 37%, partial thromboplastin time 40 sec, euglobulin lysis started after 2.5 h, fibrinogen 300 mg/dl and fibrinogen degradation products 40 µg/ml (control 2.5 µg/ml). Serum bilirubin was 2.5 mg/dl (mostly indirect), SGOT 110 mU/ml (normal $\leq$ 45 mU/ml), alkaline phosphatase 580 mU/ml (normal $\leq$ 85 mU/ml), γ-guanosine 5'-triphosphate 56 mU/ml (normal $\leq$ 30 mU/ml) and lactate dehydrogenase 1,320 mU/ml (normal $\leq$ 400 mU/ml). All other biochemical and serological studies were within normal limits and repeated blood, sputum and urine cultures were negative. Chest X-ray, liver, spleen and bone scans (T-4931) and skeletal survey were noncontributory. Three bone marrow biopsies from different areas of the iliac crests revealed similar findings (fig. 1). There was an obvious disruption of the normal marrow architecture with widespread tissue necrosis. No tumor cells were seen.

After short partial response to high dose dexamethasone and appropriate supportive therapy with platelets, red blood cells and fresh frozen plasma transfusions, the patient condition deteriorated rapidly. Marked thrombocytopenia (15,000/mm³) developed, neurologic manifestations progressed and the patient died 6 weeks after her first hospitalization.

Autopsy revealed recent subdural hematoma in the posterior and middle fossae. The gastrointestinal tract and urogenital system were normal. The lungs were mildly edematous. There was mediastinal and abdominal lymphadenopathy. Careful macroscopic and microscopic examination did not reveal primary malignant focus, but sections from the bone marrow, lymph nodes, liver, spleen and lungs revealed metastatic poorly differentiated carcinoma (fig. 2).

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Bone Marrow Necrosis as the Only Manifestation of Disseminated Carcinomatosis

Haim Granot Aaron Polliack and Yaacov Matzner

Departments of Hematology and Medicine B Hadassah University Hospital, Jerusalem, Israel

Key Words. Bone marrow necrosis carcinomatosis

Abstract. An unusual case with thrombocytopenia and bone marrow necrosis as the only manifestation of disseminated carcinomatosis is reported. The diagnosis was first established by repeated bone marrow biopsy which revealed marked necrosis without evidence of malignancy The patient's general condition deteriorated rapidly despite therapy with steroids. Autopsy revealed disseminated metastatic adenocarcinoma, but the primary site of the tumor was not identified It is suggested that malignancy should always be excluded in patients who have bone marrow necrosis without an obvious cause

Introduction

Bone marrow necrosis is most frequently associated with bacterial infections [1] Among other associated disorders, sickle cell anemia is the most common [2] Epithelial and hematologic neoplasias have also been described in association with bone marrow necrosis [3–5] The present report describes a patient with a thrombotic thrombocytopenic purpura like syndrome caused by diffuse bone marrow necrosis. Widespread metastatic adenocarcinoma was found only at autopsy To the best of our knowledge, this represents the first case of metastatic disease whose only manifestation during life was bone marrow necrosis.

Case Report

A 57 year-old Arab female was hospitalized in another hospital because of headache, fever generalized weakness and coffee ground' hematemesis of 2 weeks duration. Intestinal obstruction was diagnosed, explorative laparotomy however revealed no abdominal findings except for a benign gastric ulcer 1 week later she was transferred to the Hematology Department of the Hadassah University Hospital because of deterioration in her general condition, mental confusion and high fever Physical examination revealed a pale slightly jaundiced but severely ill patient. Multiple generalized ecchymoses were found and examination of the ocular fundi showed bilateral papilledema, flame-shaped hemorrhages, soft exudates and venous engorgement. There was no lymphadenopathy or hepatosplenomegaly



Fig. 1. Bone biopsy showing marked marrow necrosis and lack of normal hematopoietic elements. HE × 100

The neurologic examination revealed right Babinsky sign. No other neurological signs were present. Laboratory investigations showed microscopic hematuria without proteinuria. The ESR (1 h) was 70 mm, hemoglobin 10.2 g/dl, reticulocytes 0.7%, white blood cells 12,200/mm³ with normal differential count. The peripheral blood smear showed marked anisopoikilocytosis, with normoblasts.

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tendency liver biopsy and explorative laparotomy could not be performed and the only histologic material available was obtained by closed bone marrow biopsies. However all three consecutive biopsies revealed bone marrow necrosis without the presence of tumor cells, and the diagnosis of a disseminated malignant process was only established at autopsy. To the best of our knowledge this is the first case reported to have bone marrow necrosis as the only manifestation of disseminated carcinomatosis and it is recommended that a careful search for malignancy be performed in every case with a similar clinical presentation.

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Prof. A. Polliack, MD, Department of Hematology, Hadassah University Hospital, Jerusalem (Israel)

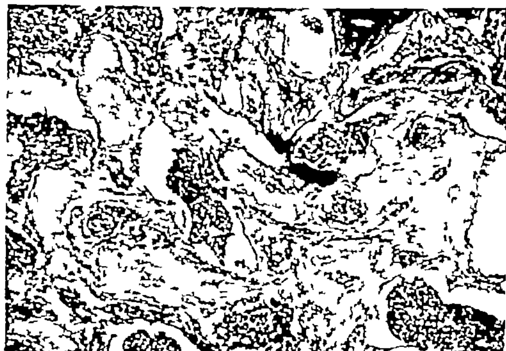


Fig. 2. Bone biopsy performed at autopsy showing metastatic adenocarcinoma involving the bone marrow HE. × 40.

Discussion

Bone marrow necrosis was *first* described in sickle cell anemia [2] and since then has been recorded with other conditions. *Brown* [1] studied 70 patients with bone marrow necrosis and showed that this finding was associated with bacterial infection in two thirds of these cases. The most common infections encountered were typhoid fever and gram-negative and positive septicemia Only one third of these patients had bone marrow necrosis with an underlying disease and if cases of sickle cell anemia are excluded the remaining cases were associated with neoplastic disorders. Of the malignant disorders described 10 were acute lymphoblastic leukemia [1 3] 6 acute myeloblastic leukemia [4] 4 malignant lymphomas [4–5] and in 10 cases bone marrow necrosis was found with a variety of carcinomas, including adenocarcinoma of the stomach, mucinous cystadenocarcinoma of ovary anaplastic carcinoma of the lung and squamous cell carcinoma of the esophagus [1 4] In 3 of the cases the primary site of the malignancy was not identified.

The patient described in this report had a poorly differentiated metastatic adenocarcinoma of unknown origin and the primary site was not found despite a careful search at autopsy However in contrast to other cases described in the literature in whom bone marrow necrosis appeared secondary to an underlying disease documented while the patient was alive the malignancy in the patient reported was not diagnosed during her lifetime The only clinical manifestations were related to a bleeding tendency resulting from thrombocytopenia and a 'thrombotic thrombocytopenic purpura like syndrome Because of the severe bleeding

tendency liver biopsy and explorative laparotomy could not be performed and the only histologic material available was obtained by closed bone marrow biopsies. However all three consecutive biopsies revealed bone marrow necrosis without the presence of tumor cells, and the diagnosis of a disseminated malignant process was only established at autopsy To the best of our knowledge this is the first case reported to have bone marrow necrosis as the only manifestation of disseminated carcinomatosis and it is recommended that a careful search for malignancy be performed in every case with a similar clinical presentation.

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Prof A. Polliack, MD Department of Hematology Hadassah University Hospital, Jerusalem (Israel)

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Idiopathic Inhibitor of Factor VIII and Its Treatment by Immunosuppression

H C Ting F Wang P Por and A Nurulhuda

Departments of Medicine and Pathology Faculty of Medicine, University of Malaya, Kuala Lumpur

Key Words. Cyclophosphamide plus factor VIII concentrate
Idiopathic inhibitor to factor VIII Immunosuppression

Abstract. A patient with idiopathic inhibitor to factor VIII is reported. Treatment with cyclophosphamide and factor VIII (given simultaneously) produced a good clinical response with disappearance of the inhibitor

Introduction

Inhibitors of factor VIII are well known in patients with haemophilia A who have received replacement therapy Rarely they have been described in post-partum females [9] in patients with collagen vascular disease, asthma, neoplasms, certain skin diseases, and drug allergy [3 9] Rarer still, they may occur in normal patients with no underlying disease [9] We report such a case and discuss the problem of treatment of this rare disorder

Case Report

A 48-year-old Chinese man was admitted in November 1977 He was well till 4 months before admission when he developed painless haematuri followed by spontaneous episodes of bleeding into his right ankle, right calf right thigh and left ankle. Physical examination on admission was normal except for pallor a swollen left ankle and ecchymosis on the right forearm. The haemoglobin was 9 4 g/dl, platelet count 420×10^9/l, total white cell count $7 1 \times 10^9$/l. The bleeding time was normal but the clotting time was prolonged. One-stage thrombin time was normal. Partial thromboplastin time was prolonged. Full thromboplastin generation test indicated factor VIII deficiency Platelet aggregation and adhesion tests were normal. The patient s plasma inactivated the factor VIII present in normal plasma, hence reveal ng the presence of a circulating inhibitor to factor VIII Subsequently the patient s factor VIII level was quantitated by the method of *Biggs* [1] and the inhibitor level determined by the *Rizza and Biggs* [1] method.

Various tests to detect any associated collagen-vascular disease and occult mal gnancies were done. The serum complements were normal. LE cells, anti-nuclear factor and rheumatoid factor were absent. The chest X ray bari m meal, barium enema, intravenous urogram, bone marrow examination, serum protein electrophoresis and immunoelectrophoresis were all normal The clinical course of the patient was stormy The sequence of events and the treatment given is illustrated in figure 1

I itially the patient was given blood transfusion and fresh frozen plasma together with high-

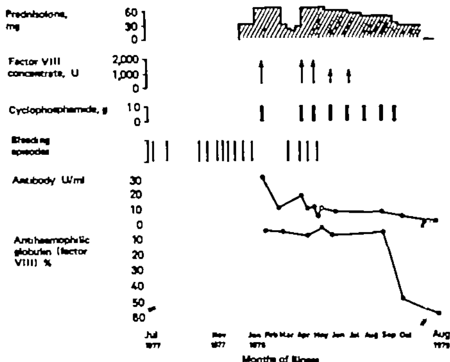


Fig. 1. Progress and treatment of patient.

dose prednisolone (60 mg/day). Despite this the patient continued to have frequent episodes of severe bleeding. Commercial factor VIII concentrate and cyclophosphamide were then given simultaneously in large doses. After the first dose (1,560 U factor VIII plus 1 g cyclophosphamide intravenously) the inhibitor level dropped dramatically and the patient was free from bleeding. However this remission was short-lived and bleeding recurred after 2 months. Four further intravenous bolus doses of the combination of factor VIII and cyclophosphamide (1 g) were given subsequently at approximately monthly intervals. The doses of factor VIII varied from 1 740 t 800 U because of difficulty in obtaining an adequate supply. After these four further bolus doses the patient stopped having any further bleeding. We then gave 1 g of cyclophosphamide alone intravenously at monthly intervals, till the patient was discharged 3 months later.

After discharge, treatment was continued with 1 g of intravenous cyclophosphamide intermittently every 2 months over another 8 months. The prednisolone (30 mg/day at discharge) was tailed off over 10 months.

At the patient's last visit he has remained free from bleeding for over year and the coagulation studies showed that the inhibitor had completely disappeared.

Discussion

The treatment of patients with idiopathic or spontaneous inhibitors is a vexing problem. In the treatment of acute bleeding episodes in these patients, factor VIII infusion can be effective in those with inhibitors in a low titre but is unlikely to be effective in patients with inhibitors in a high titre [2, 3]. To complicate matters, anamnesis may occasionally occur after factor VIII exposure

[9] In patients with high titre inhibitors non responsive to factor VIII therapy treatment with activated prothrombin complex can be successful [7] but the mechanism of action of this product is uncertain and fears have been expressed concerning thrombogenic complications [2] Exchange transfusions have so far proven to be of limited effectiveness [5]

Another approach is to attack the production of the inhibitor itself Corticosteroid therapy alone is usually not effective [1 5] Definite therapeutic benefit has occurred in some cases using 6-mercaptopurine, azathioprine, or cyclophosphamide combined with prednisolone [4 8 9] However failures have also been reported [3 9]

Dormandy et al showed that factor VIII antibody production in the Patas monkey could be suppressed if an immunosuppressive drug were given simultaneously with the factor VIII [3] *Green* [4] successfully treated a patient with factor VIII antibody by giving a combination of factor VIII and cyclophosphamide in large doses. The rationale for this mode of treatment is that the immunocytes producing the antibodies may be rendered more susceptible to the cytotoxic drug when stimulated by a large dose of antigen (factor VIII in this case) Since then, treatment modelled on this has been used on other patients with idiopathic inhibitors. This mode of treatment appears promising but failures have also been documented [5–7] and there is still uncertainty concerning the optimal dose and duration of the immunosuppressive therapy [3]

Our patient had spontaneous factor VIII antibody which was present in a high titre. Initial treatment with high dose prednisolone alone proved ineffective. When factor VIII and cyclophosphamide were given simultaneously the antibody level dropped markedly With further doses of the combination followed later by cyclophosphamide given alone the inhibitor eventually disappeared completely

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Dr H C. Ting, Lecturer in Medicine,
Department of Medicine, Faculty of Medicine,
University of Malaya,
Kuala Lumpur 22 11 (Malaysia)

Lili Fulop-Aszodi
Alloantigen Systems of Human Leucocytes and Platelets
Akadémiai Kiadó, Budapest 1979 336 pp
ISBN 963-05-1721 3

This book deals with one of the most complicated and most f scinating fields of the modern medicine the HLA system which represents the major histocompatibility system of man. The serology genetics and biochemistry of HLA determinants, as well as the practical relevance of the HLA system for various problems (organ grafting, blood transfusion, feto-maternal interactions, association with the immune response and diseases, anthropology paternity testing) are reviewed in detail Furthermore, the antigens of non-HLA systems detectable on leukocytes and platelets are discussed Unfortunat ly the interval between the termination of the manuscript (M rch 1977) and the publication of the book (end of 1979) was rather long, so that the author could not include in the text the important findings concerning the biological function of HLA gene products (g. cell cooperation or restriction phenomena) Nevertheless, the book represents valuable source of references (more than 80 pages) and can be recommended for all those who look for sound and basic information on the field of human leukocyte and platelet alloantigens.

W R Mayr

Joseph LoBue et al (eds)
Contemporary Hematology/Oncology vol 1
Plenum Press, New York 1980
XV + 330 pp US$ 29 50
ISBN 0-306-40246-7

As the Editors state in their brief introduction, the purpose of the reviews published here is to summarize investigations which have defined new concepts, and, it is hoped, stimulate new ideas' There is no doubt that the goal has been attained, since all reviews are singularly brilliant, comprehensive and provocative The subjects covered include clonality of blood cell neoplasms *(Fialkow)*, interactions of vessel alls with platelets *(Stemerman)*, heparin and complement *(Lague and Kelton)*, the molecular genetics of thalassemia *(Orkin and Nathan)*, blood group alterations in cancer *(Kahan)*, granulocyte transfusions *(Schiffer and Aisner)*, capping in lymphocytes *(Ault and Unanue)* amyloidosis *(Cohen and Cathcart)*, terminal transferase *(Marks and McCaffrey)* and kinin-releasing mechanisms in hemostasis *(Donaldson)*

Impartial treatment of review volume is rule, but also second reviewer may have his preferences Mine go to *Philip Fialkow* suggestion that at least two steps are involved in the development of chronic granulocytic leukemia, one causing clonal proliferation of pluripotent stem cells and the other inducing Ph descendants of this progenitor clone. Another provocative hypothesis offered by *Stemerman* is that material issued by the pituitary is incorporated in the platelet, and then promotes smooth cell proliferation (monoclonal) in the arteries But all articles are illuminating.

It is stated by the Editors that the subject title 'Hematology' has been changed into 'Hematology-Oncology' at reader's suggestion. Here I am resolute dissident. Regardless of the speciality in practical American medicine, to lump together ts such broad subjects would call for unbelievably broad and disperse volumes This, in fact, is pure contemporary hematology

Albert M Marmont, Genova

C R Rizza
Congenital Coagulation Disorders
Clinics in Haematology vol 8, N 1
Saunders, Eastbourne 1979
VIII + 217 pp £ 8.25
ISBN 5100-00720

This volume, the latest in the well-established series of 'Clinics in Haematology' maintains the high standard of this program for the postgraduate teaching of clinicians whose work involves haematological responsibilities Leading scientists in the field of basic and applied coagulation biochemistry *(Austen, Bangh Bidwell* and *Smith)* describe new achievements in language which is easy to follow and pleasant to read. Experienced

pathologists and clinicians exhaustively cover recent knowledge on factor VIII biosynthesis (*Bloom*) the genotype assignment and genetic counselling in the haemophilias including von Willebrand's disease (*Graham*), management of haemophilia and Christmas disease (*Biggs*), present views on von Willebrand's disease (*Nilsson* and *Holmberg*) and the clinical repercussions of fibrinogen abnormalities (*Beck*) The problem of antibodies against blood coagulation factors is updated briefly but in a masterly way (*Shapiro*) Despite the introductory remark of the guest editor (*Rizza*) that 'test tube, waterbath, and stopwatch are no longer the only means to rely upon in the diagnostic laboratory the chapter devoted to clinical laboratory investigation meant as a general introduction to laboratory diagnosis (*Lowe* and *Forbes*) does not deal with precision, accuracy and clinical relevance of the presently available and indispensable immunological and biochemical test procedures. Discussion of antithrombin III deficiency has been omitted probably because its main implications lie in thrombosis rather than in haemostasis.

In conclusion, the guest editor and authors must be complimented on this volume of the 'Clinics containing information as recent as early 1978. The 'Clinics can be recommended without reserve not only to teachers in the field of haematology and their students, but also to all scientists who are looking for a comprehensive introduction to the laboratory and clinical problems associated with congenital coagulation disorders.

E. A. Loeliger

Myron M Melamed Paul F Mullaney Mortimer L. Mendelsohn
Flow Cytometry and Sorting
Wiley New York 1979
716 pp £ 39.50
ISBN 0-471-02078-8

Among the reliable and sophisticated cell analysis systems, the techniques of flow cytometry are rapidly evolving. Because of the wide range of potential applications in biologic research and clinical medicine, however it has been rather difficult to gain an overview of this entire field. With this multiauthored book, an attempt has been made to provide a text, summarizing the different aspects of cytoflowmetric instrumentation, techniques and applications. The book is divided into various parts (a) cytophysical methods (b) cell preparation (c) cytochemical methods (d) application in cell biology immunology hematology and oncology and (e) operation systems.

Because the book covers a limited range of sophisticated techniques, and yet a very diversified field of applications, the editors have with great success improved the book by the addition of a summary chapter Through this chapter the interested reader is able to localize the type of operation system, type of methods and type of applications that has his/her special interest. Thus, the potential 'flow cytometrist' of the future has a unique opportunity on relatively neutral ground to identify and focus on the system(s) that would solve his/her problems the best. Consequently this book can help to avoid a wrong investment in expensive equipment.

The major strength of this book, however lies within the 14 chapters on application. In many impressive ways, it is clearly shown how cytochemical properties can be characterized through measurements performed on thousands of cells, in contrast to the earlier 'single-cell techniques

As many other textbooks dealing with techniques, this one might be stored on a bookshelf and rarely be consulted, but this would be a shame. First of all flow cytometry and cell sorting might soon be of the same importance to the cell biologist, as the electron microscope is for the cytologist. Secondly by learning the principles and applicability of flow cytometry a new way of thinking and creativity is automatically generated Because of the still early stage of flow cytometry the number of already interested readers might be limited. On the other hand, this book contains so many stimulating thoughts that whatever time spent on it will be quite profitable to all readers interested in cell biology cell counting and cell sorting.

Prof Dr Alain de Weck, Bern

Clinical Review

Acta haemat. 64: 241–243 (1980)

The Association of Gaucher's Disease and Dysproteinemias

Yehuda Shoenfeld, Shlomo Berliner, Jack Pinkhas and Ernest Beutler

Department of Medicine 'D', Beilinson Medical Center, Petah Tikva, Tel Aviv University Medical School, Tel Aviv, Israel, and the Scripps Clinic and Research Foundation, La Jolla, Calif., USA

The first communication of the existence of polyclonal (diffuse) hypergammaglobulinemia in Gaucher's disease was reported by *Goldfarb* et al. [4] in 1950 in a group of patients under the age of 30 years. 13 years later, the first report of a patient with Gaucher's disease and monoclonal gammopathy was published [8], followed by another report [17]. Later on, the coexistence of multiple myeloma and Gaucher's disease was described [2, 13]. *Pratt* et al. [15] described 16 patients with Gaucher's disease, of whom 6 had diffuse hypergammaglobulinemia and four monoclonal proteins, all of them of the IgG type with 'K' light chains with a concentration of the monoclonal protein of between 2,500 and 3,600 mg/dl. Except for 1 patient, none had decreased concentrations of the polyclonal protein.

The monoclonal proteins tend to appear in splenomegalic patients with Gaucher's disease above the age of 50 [15], while they are not prevalent in splenectomized patients, a fact that may be associated with the participation of the spleen in the production of these proteins.

Pratt et al. [15] suggested that patients with Gaucher's disease have continuous antigenic stimulation which brings about the development of diffuse hyperglobulinemia and later on, in elderly patients, the appearance of monoclonal proteins and even multiple myeloma [19].

Despite the small number of cases reported concerning the coexistence of multiple myeloma and Gaucher's disease, an association exists between multiple myeloma and diseases caused by other distorted lipid metabolism [1, 14, 18]. Possibly these lipids may be the antigen which induces the immunoglobulin synthesis. In effect, the repeated intraperitoneal injection of fat in BALB/c mice brought about increased production of gammaglobulins and eventually the appearance of overt multiple myeloma [9, 14].

In Gaucher's disease, there are at least three substances which may possibly serve as antigens. (1) Glucocerebroside, a lipid which accumulates in the reticuloendothelial system. (2) Glucocerebrosidase, which in patients with Gaucher's disease has a decreased activity in spite of its relatively high levels [10]. Moreover, in patients with Gaucher's disease this enzyme differs quantitatively [7] as well as structurally [10] from the enzyme present in healthy subjects. (3) Acid phosphatase, which 'leaks' into the serum following destruction of the Gaucher cells. Therefore, its concentrations is rela

pathologists and clinicians exhaustively cover recent knowledge on factor VIII biosynthesis *(Bloom)*, the genotype assignment and genetic counselling in the haemophilias including von Willebrand's disease *(Graham)*, management of haemophilia and Christmas disease *(Biggs)* present views on von Willebrand's disease *(Nilsson* and *Holmberg)* and the clinical repercussions of fibrinogen abnormalities *(Beck)* The problem of antibodies against blood coagulation factors is updated briefly but in a masterly way *(Shapiro)* Despite the introductory remark of the guest editor *(Rizza)* that 'test tube, waterbath, and stopwatch are no longer the only means to rely upon in the diagnostic laboratory the chapter devoted to clinical laboratory investigation meant as a general introduction to laboratory diagnosis *(Lowe* and *Fo bes)* does not deal with precision, accuracy and clinical relevance of the presently available and indispensable immunological and biochemical test procedures. Discussion of antithrombin III deficiency has been omitted probably because its main implications lie in thrombosis rather than in haemostasis.

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Y. Shoenfeld, MD, Department of Medicine 'D', Beilinson Medical Center, Petah Tikva (Israel)

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The identification of the antigen stimulating the production of the monoclonal component in patients with Gaucher's disease is important in light of the experimental therapy of intravenous infusion of the enzyme and its entrapment in the reticuloendothelial system [3] in these patients. The presence of monoclonal protein directed against this enzyme may interfere with the possible benefits of this enzyme.

The appearance of monoclonal gammopathy in patients with Gaucher's disease raises the question of its benign or malignant character mostly because osteolytic lesions and even pathological fractures may appear in patients with Gaucher's disease per se, with or without multiple myeloma. This problem becomes more accentuated by the recent report about pseudo-Gaucher cells in multiple myeloma [16] Thus, the diagnosis of multiple myeloma in patients with Gaucher's disease must be based upon the combination of several criteria, mainly the appearance of monoclonal gammopathy and decreased levels of polyclonal immunoglobulins [11 12] the presence of osteolytic lesions characteristic of myeloma, the presence of more than 15% plasma cells in the bone marrow most of them being immature cells, the ultrastructural characterization of these plasma cells by electron microscopy and the presence of laboratory and clinical parameters typical of myeloma and not of Gaucher's disease such as hypercalcemia hyperuricemia or kidney damage [12]

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or penicillins. Therefore, there is little justification for the use of chloramphenicol, alone or in combination, in the treatment of febrile leukemic patients. As far as the reported cases are concerned, however the beneficial effect on pyrexia may have been related to the antimicrobial activity of chloramphenicol. This drug is active against pathogens which are not sensitive in vitro or which, clinically may not respond to the combination carbenicillin-gentamicin-cephalotion (rickettsial infections, invasive salmonellosis, anaerobic infections) Combined with gentamicin, chloramphenicol has been used occasionally with good results in patients with severe infections not responding to penicillins or cephalosporins [2]

Chloramphenicol has been known for many years to reversibly suppress hemopoesis and rarely to cause irreversible aplastic anemia. Reversible bone marrow suppression is a pharmacological effect and results from inhibiton of mitochondrial protein synthesis. The mechanism of chloramphenicol induced bone marrow aplasia is not yet fully understood In vitro, chloramphenicol inhibits the DNA synthesis of marrow cells, as can be measured from the incorporation of radioactive thymidine. Although concentrations above 100 μg/ml are needed to significantly inhibit DNA synthesis in the marrow of normal subjects, this effect could be achieved with 'therapeutic' concentrations of the drug in the marrow of patients recovering from aplastic anemia. Based on these observations, it has been suggested that chloramphenicol-induced aplastic anemia may be related to a preexistent but undetected marrow abnormality [3]

Also, marrows from patients with abnormal hemopoiesis, in particular leukemic marrows, are more susceptible to the chloramphenicol inhibition of the DNA synthesis. This makes chloramphenicol a potential antileukemic agent. The observations reported in this issue of *Acta Haematologica* are in accordance with those by *Schwarz and Firkin* [4] and suggest that chloramphenicol may reduce the leukemic cells in vivo An interesting point is that the reduction of the blast cells could be achieved with a daily dose of 2 g in the patients reported by *Klein* and co-workers. These findings must be interpreted cautiously however since the decrease in blast cells count was modest in 2 of 3 patients. In addition, the concentration of chloramphenicol used in the in vitro studies were higher than those which would be achieved with daily doses of 2 g. Of course, these interesting but anecdotal observations do not prove the clinical efficacy of chloramphenicol and do not support the conclusion that it 'may have a greater role in the routine multidrug treatment of patients with leukemia Its cytotoxic action remains a life-threatening side effect of chloramphenicol and, at present, there is no justification for the use of this drug, alone or in combination, in the treatment of leukemia.

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Editorial

Acta haemat. 64 244–245 (1980)

Fever and Chloramphenicol in Leukemic Patients

Fever in leukemic patients is often due to infection, especially when they are granulopenic. Since sepsis may become overwhelming and rapidly fatal in this situation, empirical antibiotic therapy has to be started without delay in the absence of obvious noninfectious causes for the fever. Initial therapy should cover the most likely pathogens, i.e., gram negative rods (in particular *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* sp.) and *Staphyloccus aureus*. The combination of an aminoglykoside together with a Pseudomonas-active penicillin has been shown to offer an adequate coverage of these pathogens, to be efficacious even in granulopenic patients and to lack relevant toxicity [1]. Other pathogens like protozoa, fungi, mycobacteria and viruses may be encountered in leukemic patients, but they are less common and less apt to cause overwhelming sepsis. In addition, they often require the use of toxic drugs. Thus, the initial empiric treatment will usually not cover these pathogens.

In about 30% of the patients, fever will persist in spite of the antibiotic treatment. In some of these patients, the infectious cause of the fever can be documented, and treatment has to be adjusted according to the isolated organism and/or granulocyte transfusions have to be considered. In most patients who fail to respond to the antibiotics, however, no infection can be documented microbiologically or clinically. The management of fever of undetermined origin is extremly difficult in leukemic patients. The differential diagnosis includes bacterial infections resistant to antibiotics, infections due to pathogens other than bacteria and tumor fever. In particular, invasive fungal disease has to be considered in this situation. If the patient appears stable and nontoxic, most authorities recommend to discontinue the antibiotics after 5–7 days treatment and to search again for infection. In severely neutropenic patients, however, empiric amphotericin has to be considered.

In this issue of *Acta Haematologica* *Klein* and co-workers report 6 cases of fever of undetermined origin in leukemic patients which responded well to chloramphenicol. Because of a parallel decrease in the peripheral blast cell count under chloramphenicol, the authors suggest that the temperature fall was due to the cytotoxic action of this drug. Chloramphenicol is primarily an antimicrobial agent. It acts bacteriostatically and is devoid of activity against *P. aeruginosa*. In addition, this drug may antagonize the bactericidal activity of aminoglycosides



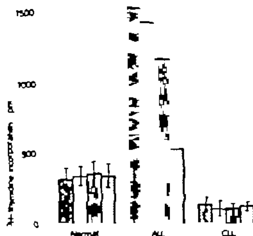


Fig. 1. The effect of CAP on the synthesizing activity of ALL, CLL and control cells.

age of blast cells in the peripheral blood decreased from 92 to 43% (table I)

Case 2

A 44-year-old woman was admitted because of fever and chills of several weeks duration. Acute myeloid leukemia was diagnosed. The hematological data are show in table I. She was treated with cytosine-arabinoside, gentamicin, cephalothin and carbenicillin for 9 days and the fever decreased, but it reappeared on day 16. CAP 2.0 g/day was administered and after 3 days the high fever decreased.

Case 3

A 23-year-old woman with acute myeloid leukemia was treated with daunomycin and cytosine-arabinoside and entered remission 2 months after induction. 1 month later she was readmitted because of fever and pains in her right knee. The hematological data are presented in table I. Blood and urine cultures were negative. She was given gentamicin 240 mg/day and cephalothin 12 g/day for 6 days, but the high temperature persisted. Since her condition progressively deteriorated, CAP 2.0 g/day was administered. After 2 days the fever decreased and the patient felt better (fig. 1).

Case 4

An 18-year-old male was diagnosed as suffering from acute lymphoblastic leukemia. Treated with vincristine and prednisone the patient entered remission. 4 months later he was readmitted because of high fever. Blood and urine cultures were negative. The hematological data are presented in table I. He was treated with gentamicin 240 mg/day, cephalothin 12 g/day and carbenicillin 30 g/day but the fever failed to decrease. CAP 2.0 g/day was administered for 6 days and the fever decreased.

Case 5

A 57-year-old male suffered from acute lymphoblastic leukemia and was treated with vincristine and prednisone. 1 year later he was readmit-

Table I. Hematological data before and after treatment with CAP

Patient	Diagnosis	Before CAP treatment			After CAP treatment		
		Hb g/dl	WBC $10^9/l$	blasts %	Hb g/dl	WBC $10^9/l$	blasts %
1	AML	9.3	7.7	92	9.0	3.8	43
2	AML	8.7	2.3	17	9.2	1.6	
3	AML	8.8	4.0	10	8.5	2.9	1
4	ALL	7.6	7.4	4	8.0	1.9	–
5	ALL	7.6	19.0	–	8.0	4.6	
6	AML	10.5	19.6		10.0	12.4	–

Original Papers

Acta haemat. 64: 246–250 (1980)

Beneficial Effect of Chloramphenicol on Pyrexia in Patients with Acute Leukemia

B. Klein, H. Bessler, U. H. Lewinski, A. M. Cohen and M. Djaldetti[1]

Department of Medicine 'B' and Hematology Clinic, Hasharon Hospital, Petah Tiqva, and Tel Aviv University Medical School, Tel Aviv, Israel

Key Words. Acute leukemia · Chloramphenicol · Pyrexia

Abstract. 6 patients with acute leukemia are described. All of them suffered from high temperature and malaise and showed negative urine and blood cultures. High doses of gentamicin, cephalothin and carbenicillin failed to lower the fever. The temperature became normal after administration of chloramphenicol (CAP) 2.0 g/day. In 3 out of 6 patients the peripheral blood blast cell count decreased following CAP administration. Incubation of acute lymphoblastic leukemia cells with CAP in vitro showed a marked decrease in the DNA synthesizing activity of the leukemic cells. The role of CAP as an additional tool in the treatment of acute leukemia is discussed.

Chloramphenicol (CAP) is a bacteriostatic antibiotic effective in a variety of infections [6]. Its side effects involve mainly the hematopoietic system [7, 8]. *Yunis* [8] described bone marrow depression during treatment with CAP. *Saidi* et al. [4] reported a greater degree of suppression in patients with disturbed hematopoiesis.

We had the opportunity to observe a beneficial effect of CAP in 6 patients with acute leukemia in whom the antibiotic was the only agent able to affect the fever and malaise, and even to cause, in some of the patients, a decrease in the number of immature cells in the peripheral blood.

[1] Established Investigator of the Chief Scientist's Bureau, Ministry of Health, Israel.

To attest further the influence of CAP on leukemic cells, we examined the effect of this drug on the DNA synthesizing activity of normal and leukemic cells.

Case Reports

Case 1

A 55-year-old male with acute myeloid leukemia was admitted 1 ½ years after the diagnosis, because of fever, chills and malaise. The white blood cell count was 7.7 × 10⁹/l with 92% blasts. Repeated blood cultures were negative. Treatment with gentamicin 240 mg/day, cephalothin 12 g/day and carbenicillin 30 g/day was instituted, but high fever persisted. Following our past experience, CAP 2 g/day was administered and subsequently the fever decreased (fig. 1). The percent

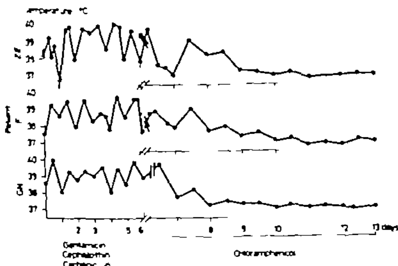


Fig. 2. The effect of CAP on the temperature of 3 patients with acute leukemia.

In 3 out of the 6 patients in the present work, a decrease in the percentage of blast cells in the peripheral blood was observed after treatment with CAP. It is conceivable to assume that CAP exerted its action on the bone marrow through reduction of the DNA synthesizing activity and subsequent decrease of the intracellular protein synthesis expressed by the reduction of the fever caused by the neoplastic process. The improvement in both fever and white blood cell count was achieved with conventional doses of the antibiotic.

CAP affects microorganisms by binding with 50S ribosomal subunit, thus inhibiting protein synthesis. *Yunis* [8] has shown that CAP inhibits DNA synthesis in normal bone marrow cells. This effect is more pronounced in leukemic cells [8]. *Schwartz and Firkin* [5] reported on a patient with chronic myelocytic leukemia in blastic crisis treated with high doses of CAP up to 12 g/day. A marked reduction in the white blood cells and blasts was observed. When the leukemic bone marrow cultures of the patient were incubated with CAP a marked reduction in DNA synthesis was noted. Our observation is in accordance with those of *Schwartz and Firkin* [5], except that the results were achieved with conventional doses of CAP.

Howell et al. [3] have shown that CAP exerts an effect similar to that of 6-mercaptopurine on normal colony forming cells in agar cultures. Therefore, CAP can be considered as a cytotoxic agent and may have a greater role in the routine multidrug treatment of patients with leukemia.

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ted because of fever Blood and urine cultures were negative. The white blood cell count was 19.0×10^9/l. He was treated with gentamicin 240 mg/day cephalothin 12 g/day and carbenicillin 30 g/day for 6 days, but the fever persisted. On the 7th day CAP was administered and after 3 days the fever decreased to normal. There were no blasts in the peripheral blood during the febrile episode.

Case 6

A 51 year-old male, with acute myeloid leukemia, was treated with daunomycin and cytosine-arabinoside. 3 months later he was admitted because of persistent fever Blood and urine cultures were negative The hematological data are shown in table I. Gentamicin 240 mg/day cephalothin 12 g/day and carbenicillin 30 g/day were given for 6 days, but the fever remained high. CAP 2.0 g/day was administered, and on the 8th day the fever decreased (fig 1) There were no blasts in the peripheral blood during the febrile episode.

Materials and Methods

Cell Preparation. Lymphocytes were separated from the peripheral blood of healthy volunteers, 1 patient with acute lymphoblastic leukemia (ALL) (case 4), and 5 patients with chronic lymphatic leukemia (CLL), using Ficoll-Hypaque density gradient. The lymphocyte-rich layer was aspirated, diluted twice in phosphate-buffered saline (PBS) and sedimented at 200 g for 10 min. Th supernatant was discarded and the sediment resuspended in PBS This procedure was repeated twice and the final lymphocyte pellet was suspended in RPMI 1640 (Gibco).

Incubation Procedures. 1 ml of cell suspension containing 2.5×10^6 lymphocytes was incubated for 60 min without and with different concentrations of CAP At the end of the incubation period, 5 μCi (methyl ^{3}H)-thymidine (50 Ci/mmol, Amersham England) were added f r an additional 120 min. The incubation was carried out at 37 °C in a humidified atmosphere containing 5% CO_2. The reaction was stopped by addition of 9 ml cold 0.9% sodium chloride solution. Further procedures were identical to those described previously [1 2] All examinations were carried out in duplicate. Blank values for nonspecific findings of radioactive material at 0 C were subtracted.

CAP (Abic, Ramat Gan, Israel) was dissolved in RPMI 1640 and was added to the lymphocyte suspensions at the following concentrations: 50, 100 and 200 μg/ml

Results

Peripheral blood lymphocytes from the ALL patient showed a 378% increase in DNA *synthesis* (fig. 2) compared to normal or CLL lymphocytes. A marked decrease in DNA synthesis was found after *incubation* of ALL lymphocytes with CAP This effect was shown to be dose-dependent with maximal inhibition after incubation with 200 μg/ml. Incubation of normal and CLL lymphocytes with CAP at the same concentration had no effect on DNA synthesis.

Discussion

The cause of the high fever in the described leukemic patients remained unclear There were no clinical findings suggestive of superimposed infection Repeated blood and urine cultures were negative Moreover the high temperature failed to respond to intensive antibiotic therapy Therefore, the leukemic process remained the only possible explanation for the continued pyrexia.

The peripheral blood cells of the patients with ALL, which were mainly blasts, showed a greater synthesizing activity in comparison to normal cells After incubation with CAP a marked reduction in the synthesis of DNA was observed in the malignant cells, while the normal cells remained unaffected. The synthesizing activity of the CLL cells was low in comparison to normal cells.

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Topographical Localization of Intracellular Immunoglobulins in Hairy Cells by Immunoelectron Microscopy

Massimo F. Martelli, Brunangelo Falini, Antonio Tabilio and Franco Aversa

Department of Clinical Medicine, Perugia University Medical School, General Hospital, Monteluce, Perugia, Italy

Key Words. Hairy cells · Immunoelectron microscopy · Immunoperoxidase · Intracytoplasmic immunoglobulins

Abstract. Ultrastructural characterization and localization of intracellular immunoglobulin in peripheral blood mononuclear cells from a patient with hairy cell leukemia were studied by the direct immunoperoxidase technique. Approximately 20–30% of hairy cells stained positive for γ-heavy and *K*-light chains, and this staining was limited to certain rough endoplasmic reticulum profiles. These ultrastructural findings can be considered an additional argument for the B cell origin of hairy cells.

Although hairy cell leukaemia (HCL) is a well-recognized clinicopathologic entity, much controversy exists concerning the lymphocytic [3] or monocytic [19] origin of the hairy cell (HC). However, most recent studies suggest that HC are derived from a subset of B lymphocytes with unusual properties such as phagocytic activity [6, 21].

In the present study we demonstrate the presence of IgG-K immunoglobulin inside the rough endoplasmic reticulum (RER) of HC by an immunoelectronmicroscopic procedure. These ultrastructural findings are evidence that HC have the capacity to synthesize immunoglobulin and support the hypothesis that HCL may be basically the result of a monoclonal B cell proliferation.

Materials and Methods

The clinical picture of massive splenomegaly without lymphadenopathy and large number of circulating neoplastic cells (30,000/mm³) with hair-like projections supported the diagnosis of HCL in our patient. Most of the cells contained tartrate-resistant acid phosphatase. HC were obtained from the peripheral blood by Ficoll-Hypaque gradient centrifugation. The population consisted of 90–100% HC. Membrane-bound immunoglobulins (SIg) were identified by direct immunofluorescence using polyvalent and monospecific *K* and *λ* rabbit antihuman immunoglobulin labeled with fluorescein isothiocyanate (Cappel Laboratories). Monospecific sheep $F(ab')_2$ antibody fragments (Nordic Diagnostic Laboratories) were used for identification of γ, μ, and δ determinants.

Immunoelectronmicroscopic studies were car-

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B Klein, Department of Medicine 'B
Hasharon Hospital, Petah-Tiqva (Israel)



Fig. 2. Immunoperoxidase electron microscopy of hairy cells. Note the electron-dense reaction products on the cell surface and in some profiles of RER. Bar = 0.1 m N counterstain. 63,840

noted in the perinuclear space or within cell organelles involved in endocytosis (fig 1). Randomly dispersed electron-dense deposits were observed on the surface of all cells which stained positively for internal immunoglobulin (fig. 2)

In control experiments in which HC were incubated with either peroxidase-labeled normal rabbit Fab fragments, free peroxidase, or DAB/H_2O_2, no reaction product was found either in the cytoplasm or on the surface of the leukemic cells.

Discussion

The demonstration of a monoclonal immunoglobulin on the surface [1] and/or in the cytoplasm [20] of HC cannot be considered as conclusive evidence of their B lymphoid origin, because the fluorescein-labeled antibodies can bind to membrane Fc receptors and to cytoplasmic IgG eventually phagocytized by the neoplastic cells. The latter phenomenon has also been reported to occur in Reed-Sternberg cells [14] and in a few cases of myelomonocytic leukemia with associated paraproteinemia [2, 11]

However the reappearence of anti-immunoglobulin surface fluorescence after trypsinization [5] and the presence of immunoglobulin in the culture medium [7 10 18] demonstrated in recent in vitro studies, suggest a B cell origin for HC. Moreover in at least _ cases of HCL with associated paraproteinemia [4 8] the type of surface and cytoplasmic immunoglobulin was identical to the serum M component.

In the present study an immunoperoxidase technique was employed to provide information on the ultrastructural distribution of intracellular immunoglobulin in HC. Our results show that the positivity in the RER cisternae is spotty and irregular and this suggests the presence of a small quantity of IgG-*K* immunoglobulin A similar immunoelectron microscopic pattern has also been observed in the cytoplasm of normal lymphocytes [12]

The possibility that in our case the RER staining was related to endogenous peroxidase activity was ruled out by the following findings. (a) endogenous peroxidase activity was absent on the HC surface but present in both the RER and the perinuclear space [17] (b) HC peroxidase was inhibited by the routine fixation method used [17] (c) the pellets incubated with peroxidase-labeled normal rabbit Ig Fab fragments, free peroxidase, or DAB/H_2O_2 showed no positive reaction.

The HC phagocytic capacity [9] would seem to indicate that the intracytoplasmic immunoglobulin may be exogenous in origin. However the localization of electron-dense deposits in the RER, the cellular site

ried out with monospecific rabbit antibodies against γ, Λ and λ chains (Cappel Laboratories) reduced to monovalent Fab fragments according to the method of *Porter* [15]. The Fab fragments were then coupled to horseradish peroxidase (HRP) by the method of *Nakane and Kawaoi* [13] and separated from unconjugated Fab and enzyme by chromatography on Sephadex G-100. Normal rabbit monovalent Fab fragments (Nordic Diagnostic Laboratories) conjugated with HRP were used for control experiments.

Samples of 10^7 cells were washed three times in Hanks' balanced salt solution at room temperature. The cell pellets were then fixed by resuspension in 1.25% glutaraldehyde in PBS (pH 7.4 0.15 *M*) for 30 min and twice washed in the same buffer [16]. An excess (80 µl) of HRP-labeled Fab fragments (1 mg/ml) was added to the pellets, and the samples were left for 1 h at 0 °C. After three washes in PBS the cells were incubated with diaminobenzidine (DAB) in 0.1 *M* Tris-HCl pH 7.6 for 30 min followed by DAB Tris-HCl containing 0.01% H_2O_2 for another 20 min. They were post fixed with 1% osmium tetroxide for 30 min, washed, dehydrated in alcohols and embedded in Epon. Ultrathin sections were cut on a Reichert ultramicrotome and examined without counter stain with a Phillips electron microscope.

The following controls were used: (a) substitution of conjugated Fab of rabbit antihuman immunoglobulins by conjugated normal rabbit immunoglobulins (Fab fragments); (b) incubation in HRP alone (1 mg/ml), and (c) detection of endogenous peroxidase in DAB medium without prior incubation with peroxidase-labeled antibodies.

Results

95% of HC exhibited extremely bright surface fluorescence with a polyvalent anti immunoglobulin antiserum and, when immunoglobulin class-specific fluorescent antisera were used 90% of HC were stained by both anti-γ and anti-*K* antibodies. No reactivity was found with antisera to IgA, IgM, IgD and λ light chain. The resynthesis of surface IgG Λ was evident in the majority of HC after trypsinization and culture.

In immunoelectron microscopy varying numbers of HC (about 20–30%) stained positively for γ-heavy and Λ light chains. No reactivity was found with anti λ antibody fragments. The localization of the electron-dense reaction product was restricted to only some RER profiles; no positivity was



Fig. 1a, b. Hairy cells incubated with rabbit Fab-peroxidase conjugate antihuman γ-chain. Black reaction product present in RER strands. Bar = 1 µm. No counterstain. × 25,000.



Fig. 2. Immunoperoxidase electron microscopy of hairy cells. Note the electron-dense reaction products on the cell surface and in some profiles of RER. Bar = 0.1 μm. No counterstain. 63,840

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The HC phagocytic capacity [9] would seem to indicate that the intracytoplasmic immunoglobulin may be exogenous in origin. However the localization of electron-dense deposits in the RER, the cellular site

of protein synthesis, and their complete absence in lysosomes seem to exclude this possibility In addition, the presence of the same IgG-λ immunoglobulin in the cytoplasm and on the surface of HC suggests that it is being produced by these cells themselves.

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Massimo F. Martelli, MD, Istituto di Clinica Medica Generale e Terapia Medica, Università di Perugia, Policlinico, Monteluce, 06100 Perugia (Italy)

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On the Inhibitory Effect of the Serum of Uraemic Children on Erythropoiesis

P Scigalla V I Gudim V S Ivanova N A Fedorov and P Grossmann

Research Department of Pediatric Clinic, Humboldt University Berlin, and
the Central Institute of Haematology and Transfusion Service of the USSR, Moscow

Key Words. Erythropoiesis Erythropoiesis inhibiting factor Erythropoietin Haemodialysis Uremia

Abstract. The erythropoietin activity and the erythropoiesis inhibiting factor(s) (EIF) were studied in the serum of 10 children and adolescents suffering from terminal renal insufficiency The influence of haemodialysis on these factors was examined as well As a test model for the estimation of erythropoietin activity and EIF we used hypertransfused polycythaemic mice. No erythropoietin activity was detectable in the serum of children and adolescents. A 40%-inhibitory effect on erythropoiesis existed in 8 out of 10 uraemic sera prior to dialysis After haemodialysis the inhibition is eliminated in 4 of the 9 sera examined, and reduced to half in the 5 remaining sera. The findings suggest that the inhibitory effect is possibly caused by one or several EIF of the middle molecule group

The erythropoiesis is influenced by stimulatory and inhibitory factors. The most important activator of erythropoiesis is erythropoietin (ESF) This hormone is a glycoprotein with a molecular weight of about 41 000 which acts on the differentiation of the erythroid stem cells [23 24 44 48] The main site of ESF production is the kidney [14 19]

So far there is only little and contradictory information available on the inhibitory factors of erythropoiesis Inhibitory factors were found in the serum of polycythaemic animals after transfusion [15 27 31 51] in highlanders that were rapidly moved down to sea level [41 45] and in the plasma and urine of newborn children [8 28 36] These factors probably act at the level of the erythropoietin responsive cells [32, 48] either as antagonist to ESF or as a chalone [5 29 46]

In patients with chronic renal insufficiency the erythropoiesis is disturbed considerably A hyporegeneratory anaemia develops with an increasing restriction of kidney function. There exists a close relationship between the degree of kidney insufficiency and the degree of anaemia [19 38 49] Reduction of ESF production is certainly one of the causes of renal anaemia This situa

tion is stressed by the fact that in the majority of the patients after renal transplantation, ESF can be found in the urine again, erythropoiesis increases, and the anaemia improves [13 40] *Van Dyke et al.* [52] did not find any increase of reticulocytes after injection of ESF in patients with terminal renal insufficiency while a reticulocytosis occurred in healthy persons after an ESF dosis that was three times lower *Essers et al.* [12] showed that the ESF dosis necessary for a reticulocytosis in uraemic patients depends on the degree of uraemia. These results indicate that the sensitivity of the bone marrow to ESF is diminished in uraemia.

The inhibitory effect of uraemic serum on erythropoiesis was seen in a number of investigations [4 11 16–18, 34 41, 42, 50] A reduced proliferative activity of erythroblasts in cultures was found by *Brunner et al* [6] and *Markson and Rennie* [37] *Markson and Rennie* [17] showed a reduction of ³H-thymidine 2-desoxyriboside incorporation in tissue cultures. Others [17 25 42, 50] found an inhibition of haem synthesis in tissue cultures after an addition of uraemic serum.

The causes leading to the inhibition of erythropoiesis by uraemic serum are not yet clearly known. It may possibly be due to the accumulation of uraemic metabolic products and/or of the erythropoiesis inhibiting factors (EIF) as a consequence of reduced excretion [34 42]

Using the bioassay system of the polycythaemic mouse, which is specific for the determination of ESF [14 22] *Fisher et al.* [16] found an inhibitory effect in 5 out of 6 uraemic sera after application of ESF *Essers et al.* [12] was unable to confirm this inhibitory effect in an analogous biological assay system in the serum of 26 uraemic patients. Apart from these few statements there are no investigations of the EIF in the serum of renal insufficient children and the influence of haemodialysis on its activity Therefore, we determined the ESF and EIF in serum of children and adolescents who are in a chronic haemodialysis programme.

Materials and Methods

We examined the serum of 10 children and adolescents suffering from terminal renal insufficiency in the chronic haemodialysis programme. The children were dialysed 3 × 3 or 3 × 4 h/week, respectively by capillary dialyser Cordis Dow HFAK 4 (1.3 m surface). The uraemic serum was extracted immediately before and after haemodialysis, and stored at –20 °C until examination.

Table I lists the general clinical and anamnestic data. The average age of the patients was 14 1 years (9–17 years), the average number of dialyses/child was 117 (2–444). All patients had distinct anaemia with mean haemoglobin concentration 6.1 g%, haematocrit 18 vol%, and reticulocytes 20‰ or 0.8%, respectively when the reticulocyte index was calculated according to *Gansoni* [21] 9 of the 10 children received an average of 3 4 transfusion units (300 ml washed erythrocyte concentrate each) during the preceding 9 months. Patient K. Kos, the only child subject who had been binephrectomised, received 13 transfusions during the same period. The serum creatinine and urea N values varied around mean of 11.. and 90.5 mg/100 ml, respectively Only patient K. Kos. was anuric. For the other patients the 24-hour amount of urine varied between 30 and 400 ml.

Bioassay System

As test model we used polycythaemic female mice of the Swiss-Webster strain ranging between 22 and 27 g body weight. They were allowed to feed and drink *ad libitum*. Polycythaemia was achieved by hypertransfusion of heterologic blood that had previously been washed and adjusted to Hk of 70 vol% using 0.9% NaCl solution. The mice were transfused with 0.9 ml blood i.p. on the 1st day and with 0.8 ml i. on the 2nd day On the 8th day post-transfusion the mice had mean

Table I. Diagnostic and haematologic values of children and adolescents with terminal renal insufficiency

Patient	Age, years	*Renal disease*	*Haemoglobin-concentration, g%*	*Haema-tocrit vol%*	*Reticulo-cytes %*	*Number of transfusions during the preceding 9 months*	*Creatinine mg%*	*Urea mg%*
I Gr	17	chronic glomerulo-nephritis	5.5	15	20	2	18.5	144
R. Be.	9	chronic glomerulo-nephritis	5.8	18	18	6	7 0	72
A. Ru.	14	chronic glomerulo-nephritis	6.0	19	24	3	12.8	78
G Gu.	15	chronic glomerulo-nephritis	7.3	20	20	0	10.0	78
K. Ko.	16	chronic pyelo-nephritis	6.4	20	18	13	10.5	75
U Er	16	chronic pyelo-nephritis	8.5	24	34	0	13.0	67
U Ec.	13	chronic pyelo-nephritis	5.2	16	20	4	11 0	100
A. Wl.	17	nephrophthisis	4 8	12	20	6	9.9	81
D Ri.	10	nephrophthisis	5.5	16	10	8	13.0	129
F Ma.	14	nephrophthisis	5 0	17	16	4	8.0	98
			6.0 ± 1 1	17 7 ± 3.3	20.0 ± 6.1	4 6 ± 3.9	11 4 ± 3.2	91 4 ± 25 7

haematocrit level of 62 vol%. Mice with a Hk below 55 vol / were not used.

Erythropoietin Estimation. Mice were injected 0.5 ml serum i.p each on the 4th and 5th day post-transfusion. On the 6th day 0.5 μCi ^{59}Fe were injected i.v The blood samples were used for the determination of Hk values, reticulocyte counts, and 0.5 ml of blood for the ^{59}Fe measurement. A group of mice with injections of 0.9% NaCl solution served as control.

EIF Estimation. Polycythaemic mice were injected 0.5 ml of uraemic serum i.p each, and 0.25 IU ESF s.c. each on the 4th and 5th day post-transfusion. The ESF which had been purified from the urine of patients with aplastic anaemia [14] was calibrated against International Standard B (Division of Biological Standards, National Institute for Medical Research, Dollis Hill London). As for the ESF estimation the mice were also bled on the 8th day post-transfusion and the Hk, reticulocytes and the rate of the Fe incorporation into the red blood cells were determined. The results in those mice which had received injections of test material plus ESF were compared with those of mice which had received injections of NaCl solution plus ESF The difference indicates the degree to which erythropoiesis was inhibited [14 35]

Results

Table II shows the ESF level of the uraemic serum samples expressed in ^{59}Fe incorporation rate in red blood cells of polycythaemic mice. The rate of ^{59}Fe incorporation was 0.26 ± 0 12%.

Table III shows the rates of ^{59}Fe incorporation and reticulocyte counts in blood of polycythaemic mice, which were inject

Table II. Erythropoietin activity in uraemic serum of children and adolescents, expressed as the ^{59}Fe incorporation (± SE) into the red blood cells in hypertransfused polycythaemic mice

Patient	% ^{59}Fe uptake in the RBC
Saline	0.14 ± 0.04
I. G.	0.23 ± 0.18
D. Ri.	0.29 ± 0.28
K. Kos.	0.27 ± 0.19
A. Wi.	0.28 ± 0.10
F. Ma.	0.12 ± 0.10
U. Er.	0.35 ± 0.20
A. Ru.	0.08 ± 0.03
U. Ec.	0.44 ± 0.12
n = 8	0.26 ± 0.12

ed ESF and the serum of 10 children and adolescents with terminal renal insufficiency before and/or after haemodialysis, and from 2 serum samples of normal persons.

While no inhibitory effect could be seen in the 2 normal serum samples we found an inhibitory effect in 8 out of 10 uraemic serum samples taken immediately before haemodialysis. The inhibition of ^{59}Fe incorporation was on the average 40%, the reticulocyte count 45% (fig. 1). There is a particularly high inhibitory effect in patient K. Kos. who had been binephrectomised with 67% of ^{59}Fe incorporation and 95% inhibition of reticulocytes. No inhibitory effect was found in patients R. Be. and D. Ri. In addition, table III shows the influence of haemodialysis on the inhibitory effect of the uraemic serum. Of the 9 samples taken immediately after haemodialysis only 5 showed an inhibitory effect while no difference to the control group could be found in 4 sera. The average inhibitory effect was 27% related to the ^{59}Fe incorporation and 20% related to the reticulocytes. This suggests that a clear reduction of inhibitory effect of uraemic serum could be obtained by haemodialysis (fig. 2). There is a close correlation (r = 0.92) between the rate of ^{59}Fe incorporation and the reticulocyte

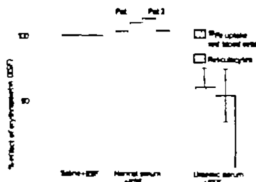


Fig. 1. Inhibitory effect on the ^{59}Fe incorporation into the RBC and the reticulocytes in hypertransfused polycythaemic mice, injected with uraemic serum from children and adolescents.

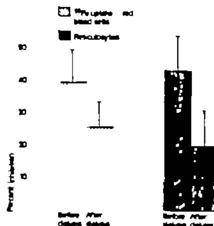


Fig. 2. Influence of the haemodialysis on the inhibitory effect of uremic serum on the ^{59}Fe incorporation into the RBC and reticulocytes in hypertransfused polycythaemic mice.

Table III. Effects of serum of uraemic and normal patients on the erythropoietin activity in polycythaemic mice, expressed as the ^{59}Fe uptake into the RBC and reticulocytes

Material	Dosis		% ^{59}Fe uptake			Reticulocytes, ‰		
	ESF IE	serum ml	x ± SD	% inhibition	p	x ± SD	% inhibition	p
Normal serum								
Saline	–	–	0.41 ± 0.4 (5)	–	–	0.2 ± 0.1 (5)	–	
Saline + ESF	0.5	–	7.81 ± 2.5 (10)	–	–	12.63 ± 3 1 (10)	–	
Normal serum 1	0.5	1.0	7.56 ± 1 7 (5)	–	–	13 6 ± 3 0 (5)	–	
Normal serum 2	0.5	1 0	8.84 ± 2.6 (5)	–	–	12.0 ± 3.3 (5)	–	
Uraemic serum								
Saline	–	–	0.14 ± 0.04 (6)	–	–	4 0 ± 1 4 (4)	–	
Saline + ESF	0 8	–	5.94 ± 1 7 (5)	–	–	17.0 ± 4.2 (7)	–	–
Pat. K. Kos. b. h.	0 8	1.0	1.98 ± 1 1 (4)	67	< 0.01	1 0 ± 0.4 (5)	95	< 0.01
Saline	–	–	0.21 ± 0 1 (4)	–	–	0.75 ± 0.2 (4)	–	–
Saline + ESF	0.8	–	5.28 ± 1.5 (5)	–	–	18.3 ± 3 6 (6)	–	
Pat. F Mac. b h.	0.8	1.0	3.50 ± 0 7 (5)	34	< 0.01	9.2 ± 2.9 (5)	50	< 0 01
Pat. R. Be. b. h.	0.8	1.0	4.32 ± 2.4 (5)	18	n.s.	13 4 ± 2.6 (5)	27	n.s.
Saline	–	–	0.35 ± 0 4 (6)	–	–	0.33 ± 0.4 (5)	–	–
Saline + ESF	0.8	–	7.86 ± 1.5 (5)	–	–	15.9 ± 3 1 (7)	–	
Pat. U Eck. b. h.	0.8	1.0	5 75 ± 1 7 (5)	27	< 0.02	6.6 ± 0.5 (5)	58	< 0 01
Pat. U Eck. p. h.	0.8	1.0	5 65 ± 1 7 (3)	28	< 0.02	10.3 ± 1.5 (3)	35	< 0.02
Saline	–	–	0.36 ± 0.2 (5)	–	–	0 4 ± 0.4 (5)	–	
Saline + ESF	0 8	–	24.8 ± 3.9 (5)	–	–	18 6 ± 5 0 (5)	–	–
Pat. I. Gr b. h.	0 8	1 0	16.8 ± 4 1 (6)	82	< 0.005	11.3 ± 3 6 (6)	35	< 0.05
Pat. I. Gr p. h.	0.8	1.0	10.2 ± 3 1 (4)	60	< 0.01	13.2 ± 3 1 (5)	25	n.s.
Pat. D R. b. h.	0.8	1.0	20.6 ± 3 1 (4)	17	n.s.	21.0 ± 1 4 (4)	–	n.s.
Saline	–	–	0.48 ± 0.1 (5)	–	–	0.8 ± 0.6 (6)	–	–
Saline + ESF	0.5	–	8 49 ± 2.0 (5)	–	–	10.2 ± 2.9 (6)	–	–
Pat. G Gu. b h.	0.5	1.0	5.54 ± 2.0 (5)	35	< 0.05	6.8 ± 2.4 (5)	33	n.s.
Pat. G Gu. p. h.	0.5	1.0	4.80 ± 1 0 (4)	44	< 0.05	6.8 ± 2.2 (6)	33	< 0.05
Saline	–	–	0.41 ± 0.5 (4)	–	–	0 (4)	–	–
Saline + ESF	0.5	–	6.62 ± 1.5 (6)	–	–	9.0 ± 2.8 (5)	–	–
Pat. A. Ru. b. h.	0.5	1 0	4.23 ± 1 0 (4)	38	< 0.05	5.5 ± 2.3 (4)	39	< 0.05
Pat. A. Ru. p. h.	0.5	1.0	5 44 ± 1 6 (5)	18	n.s.	8.4 ± 2.5 (5)	10	n.s.
Pat. A. Wl. b. h.	0.5	1 0	4.02 ± 1 7 (5)	39	< 0.05	6.6 ± 2.8 (5)	27	< 0.05
Pat. A. Wl p. h.	0.5	1 0	6.9 ± 1.8 (5)	0	–	15.0 ± 3 0 (5)	0	–
Pat. U Er b. h.	0.5	1 0	2.43 ± 0.8 (5)	63	< 0.01	5 4 ± 2.0 (5)	40	< 0.01
Pat. U Er p. h.	0.5	1.0	3 4 ± 2.4 (3)	49	< 0.01	8 0 ± 2.7 (4)	12	n.s.

The values are expressed as mean ± SE. The parentheses indicate the number of mice in each assay
b h. = before haemodialysis.
p. h. = post haemodialysis.
n.s. = not significant.

count. No relationship can, however be proved between the time when the disease occured, the frequency of dialysis, the serum creatinine and BUN levels, transfusion frequency haemoglobin concentration and haematocrit on the one hand, and the inhibitory effect on the other hand.

Discussion

No ESF could be found in the serum of any renal insufficient patients. These results agree with those obtained by a great number of authors [3 18, 19 43 49] The lowered reticulocyte count reflects the hyporegeneration of the anaemia. The transfusion frequency varied considerably although no erythropoietin activity could be found in serum of the children. This situation seems to indicate that further factors also pathogenetically important, become effective for renal anaemia. The evidence of the inhibitory effect of the uraemic serum in 8 out of 10 patients taken before haemodialysis agrees with the results obtained in adults by *Fisher et al.* [16]. They found an inhibitory effect of 65% on the average of the uraemic serum taken from 5 out of 6 adult patients. *Esters et al.* [12] found no inhibitory effect in the serum of 26 patients with terminal renal insufficiency The relatively high ESF dosis applied to the mice makes recognisation of the inhibitory effect difficult and may be regarded as a possible cause of its absence.

No relationship could be proved between serum creatinine and urea N level and the degree of anaemia on one hand and the inhibition of the erythropoiesis on the other hand. Other authors also reported that there is no close relationship between creatinine and BUN respectively and the degree of the anaemia in patients with dialysis [10, 30 33 47] In contrast to our patients they found an improvement of anaemia in the dialysis treatment, in spite of unchanged serum data. *Wallner et al.* [50] observed an inhibition of haem synthesis *in vitro* with uraemic serum. There was no inhibition of haem synthesis following the addition of creatinine, urea and guanidine succinic acid.

At the end of haemodialysis a significant decrease of the inhibitory effect was observed and in 4 patients even an elimination of the inhibitory effect. This decrease of inhibition suggests that the inhibitory effect is caused by (a) dialysable substance(s) and leads to the question whether the inhibitory effect is specific and/or non-specific and by which substances it is caused. After haemodialysis a reduction of the inhibitory effect of the uraemic serum on the haem synthesis was also observed. *Moriyama et al.* [42] isolated an inhibitor of haem synthesis from uraemic serum with a molecular weight of about 2,00–5,000. This inhibitor was similar to that found in polycythaemic and normal rabbit serum. The authors suggested that the absence of the excretion of urine in the uraemic patients might be the reason for the increased inhibitor concentration in the serum [42].

The findings of *Lindeman* [35] that the inhibitory effect occurs only when the time between ESF application and serum does not exceed 6 or 12 h respectively suggest that the inhibition acts on the earlier stadium of the erythron.

The view that the inhibitor(s) belong(s) to the so-called 'middle molecules' which are accumulated in uraemia [1 2, 7 9 20 25 26, 39], is supported by a number of findings. For instance, an improvement of anaemia by extension of the weekly dialysis time was seen. In the case of patients with a haematocrit of 20 vol% which had initial-

ly been dialysed 2 × 6 h/week there was an increase of Hk to 30 vol% after 3 months when the dialysis time had been extended to 2 × 9 h/week. Since the dialysance of middle molecules is largely independent of blood flow and only depends on dialysis time, dialyser membrane and dialyser surface the anaemia may have been improved by the increased dialysance of the middle molecules.

In conclusion, we state that an inhibitory effect on erythropoiesis could be found in 8 out of 10 uraemic sera of children who underwent treatment in a chronic haemodialysis programme. This inhibitory effect was observed only in 5 out of 9 patients after haemodialysis. In these patients the inhibition was diminished by about 50% than before dialysis. The inhibition may be possible, the result of the accumulation of an erythropoiesis inhibitor that is part of the middle molecules. If this view could be confirmed by further investigations it might lead to the improvement of renal anaemia by changes in the dialysis technique and the use of new dialysers.

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P Scigalla, Bereich Medizin (Charité) der Humboldt Universität zu Berlin, Kinderklinik, Schumannstr 20/21, DDR-104 Berlin

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Bone Marrow Cellularity and Iron Stores in Chronic Renal Failure

Darrel O Ho-Yen Najma Saleem Laura W Fleming William K. Stewart and Henry B Goodall

University Department of Pathology Haematology Section, and University Department of Medicine, Ninewells Hospital, and Renal Unit, Dundee Royal Infirmary Dundee, Scotland

Key Words. Bone marrow Iron Renal failure

Abstract. Bone marrow biopsies of 15 patients with chronic renal failure (CRF) were compared with those of a control group matched for age and sex. Using a point-counting method for assessing cellularity there was no significant difference in marrow cellularity between the CRF patients and the control group There was a significant difference in the myeloid–erythroid ratio ($p < 0.05$) between the control group and the CRF patients, the latter group having a lower ratio. A point-counting method was used for the assessment of iron stores. Of 8 CRF patients who had not been given intravenous iron, oral iron or blood transfusion, 2 had greater iron stores than the control group.

Introduction

Anaemia is a common feature of chronic renal failure (CRF) A major factor in this anaemia is the inability of the bone marrow to produce enough red cells, yet bone marrow cellularity in CRF has not been adequately studied. Previous reports have been contradictory *Eschbach et al.* [1] found 50% of their series of marrows to be hypocellular whereas *Callen and Limarzi* [2] deduced that 80% of their patients had hypercellular marrows. Some of this discrepancy may be due to the difficulty in measuring marrow cellularity which, in the past, has been subject to gross error [3] Recently we have developed an accurate, objective point-counting method for this assessment in aspiration biopsies [4]

Iron stores in bone marrow biopsies are usually assessed by the subjective opinion of experienced observers with the aid of a grading system. This method is not accurate. *Bentley and Williams* [5] have shown that when four observers assessed 60 marrows independently there was unanimous agreement in only 20 marrows. Thus, it was decided to adapt our point-counting method for assessing bone marrow cellularity to measuring iron stores in the marrow This investigation compares the cellularity and iron stores of bone marrow biopsies from CRF patients with those of a control group.

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University Department of Pathology, Haematology Section, and University Department of Medicine, Ninewells Hospital, and Renal Unit, Dundee Royal Infirmary, Dundee, Scotland

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Abstract. Bone marrow biopsies of 15 patients with chronic renal failure (CRF) were compared with those of a control group matched for age and sex. Using a point-counting method for assessing cellularity, there was no significant difference in marrow cellularity between the CRF patients and the control group. There was a significant difference in the myeloid-erythroid ratio ($p < 0.05$) between the control group and the CRF patients, the latter group having a lower ratio. A point-counting method was used for the assessment of iron stores. Of 8 CRF patients who had not been given intravenous iron, oral iron or blood transfusion, 2 had greater iron stores than the control group.

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Material and Methods

Bone Marrow Cellularity

Bone marrow was aspirated from the sternum using a Salah needle. Marrow cellularity was assessed by point-counting [4]. Sections of the marrow biopsy were stained with Mayer's haemalum and eosin and mounted in synthetic resin. A grid was superimposed on the marrow preparation so that the marrow was divided into equal quadrants. A graticule (10 × 10 mm) with 121 intersection points was fitted in a 6.3 focussing eyepiece. With a × 40 objective and counting an equal number of fields from each quadrant or all of the marrow specimen, a minimum total of 600 points was counted. If the biopsy contained insufficient material for 600 points to be counted, the specimen was rejected.

Myeloid-Erythroid Ratio

A minimum of 500 nucleated cells are counted in a cellular area of the marrow smear working back from a fragment of marrow [6].

Iron Stores

Sections of the marrow biopsy were stained by Perl's Prussian blue reaction and mounted in synthetic resin. The point-counting method was adapted for counting iron stores. A graticule (10 × 10 mm) with 441 intersection points was used, the four corner intersections being ignored as with point-counting bone marrow cellularity. The area of marrow used for assessing cellularity was counted for iron stores and the result expressed as iron-positive points per 1,000 marrow points.

The reproducibility of the method was tested by counts on four marrow preparations with a range in iron stores (one from each group in table I) on six separate occasions. Co-efficients of variation for each group were = 0, 0, 25, 19%. In each case when the marrow was recounted, it remained in the same group.

Patients

Bone marrow was obtained from 27 patients with end-stage chronic renal failure. However in 12 specimens there was insufficient material for the point-counting assessment. The remaining 15 patients, 9 male (aged 19–50) and 6 female (aged 30–63 years), had CRF from a variety of causes: chronic pyelonephritis (3), chronic focal glomerulonephritis (2), diabetic nephropathy (2), polycystic kidney disease (2), nephrotic syndrome (2), chronic glomerulonephritis (1), hypertension complicated by disseminated intravascular coagulation (DIC) (1), systemic lupus erythematosus (1), analgesic nephropathy (1). None of the patients had evidence of current infection and all had a normal white cell count. 10 had been haemodialysed for periods between 2 and 71 months, 2 patients being on second periods of dialysis after a failed renal transplant. 4 of the 15 had been given intravenous iron therapy, 2 oral iron and 1 a blood transfusion. The patients' mean haemoglobin was 8.2 g/dl (range = 5.9–11.4 g/dl).

Control Group

This group consisted of 15 individuals who were matched for sex and age (within 5 years) with the CRF patients. Biopsies on these patients were made to exclude lesions of the marrow, mainly suspected neoplasia. No member of the control group was anaemic and all had a morphologically normal peripheral blood film and aspiration marrow biopsy. All had normal kidney function. The patients' mean haemoglobin was 14.1 g/dl (range = 12.1–16.1 g/dl).

Results

Bone Marrow Morphology

All of the patients and controls showed normoblastic erythropoiesis and normal morphology of the white cells and their precursors.

Bone Marrow Cellularity

In the control group, the degree of marrow cellularity was random and bore no relationship to the indication for the marrow examination. These marrows were considered to be normal. The results from the two groups were analysed by the Student's paired t test. There was no significant difference between the marrow cellularity of the patients with CRF and the control group (fig. 1). The cellularity of the CRF cases did not have any relationship to the degree

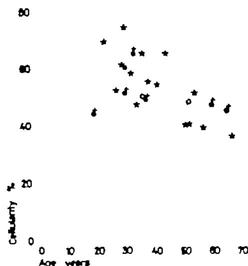


Fig. 1. Marrow cellularity vs. age. ☆ = Normals ● = CRF with dialysis; O = without dialysis; + = reduced myeloid-erythroid ratio.

of anaemia, length of dialysis or whether or not intravenous iron had been given.

Myeloid Erythroid Ratio

In the CRF patients, the mean myeloid-erythroid ratio was 3.0:1. 6 patients showed relative excess of erythroid cells (ratio < 2.5:1) (range 1.8–2.3:1) and the remainder had a normal ratio (range 2.6–5.5:1). In the normal patients, the mean myeloid-erythroid ratio was 3.7:1 (range 2.5–6.1:1). Analyses of the results by Student's paired t test showed that there was a significant difference in the myeloid-erythroid ratio ($p < 0.05$) between the CRF patients and the control group.

Iron Stores

2 of the 8 patients who had not been given intravenous iron, oral iron or blood transfusion had increased iron stores (> 8/1,000 marrow points) compared with the control group (table I). There appeared to be no relationship between iron stores and marrow cellularity. Iron stores in the normal controls ranged between 0 and 7/1,000 marrow points, with the majority between 1 and 7/1,000 marrow points.

Discussion

Bone marrow morphology has been found to be independent of the underlying disease causing CRF [7]. The discrepancies in previous reports on bone marrow cellularity are not easy to explain. *Eschbach et al.* [1] were primarily interested in ferrokinetic studies and marrow cellularity was a minor investigation, thus there is no information on their methodology. Detailed methodology and results have been found in only one previous study [2].

Callen and Limarzi [2] made two main observations from their studies on the bone marrow in CRF: firstly they deduced that 80% of their patients had hypercellular marrows, and secondly using the myeloid-erythroid ratio, they concluded that 20% of their patients had some erythroid hypoplasia. Their first observation is at variance with our results which show that there is no significant difference between the marrow cellularities of the patients with CRF and a control group matched for age and sex. As bone marrow cellularity varies with age and sex and there is a wide range in normal individuals [8], *Callen and Limarzi* [2] might have interpreted their results differently if they had compared their patients with normals matched for age and sex. In addition, they measured marrow cellularity by a visual assessment of sections relying on the haematologist's judgment. This method has

been shown by *Morley and Blake* [3] to be inaccurate and not reproducible.

The second observation of *Callen and Limarzi* [2] was that 9 of their 44 patients (20%) had a myeloid-erythroid ratio of 4:1 which was believed to represent erythroid hypoplasia. However, their ratio is within the normal range of 2.5–15:1 given by *Dacie and Lewis* [6]. Further, if it is assumed that there is no myeloid hypoplasia, 30 of *Callen and Limarzi*'s patients (68%) have a myeloid-eryhtroid ratio of under 2.5:1 and so show erythroid hyperplasia. We have found a significantly lower myeloid-erythroid ratio in CRF patients as compared with the controls.

One factor which has so far not been taken into consideration in the interpreta-

Table I. Iron stores in marrow biopsies

Underlying disease causing CRF	At time of biopsy		Number of iron points per 1,000 marrow points		
	months of maintenance dialysis	iron therapy	0	1–7	8
1 Diabetes	–	–			+
2 Diabetes	–	–		+	
3 Systemic lupus erythematosus	–	–	+		
Total (non-dialysed patients)			1	1	1
4 Chronic focal glomerulonephritis	2	–		+	
5 Chronic pyelonephritis	2	–	+		
6 Hypertension + DIC	3	–	+		
7 Nephrotic syndrome	4	–		+	
8 Chronic pyelonephritis	6	–			+
Total (dialysed patients)			2	2	1
Total (patients not given iron)			3	3	2
9 Polycystic kidneys	–	transfusion 7 units 6/12 earlier			+
10 Chronic pyelonephritis	–	oral iron 4/12	+		
Total (non-dialysed patients)			1	0	1
11 Polycystic kidneys	4	oral iron			+
12 Nephrotic syndrome	18	8/12 after i.v. iron			+
13 Chronic glomerulonephritis	26	16/12 after i.v. iron	+		
14 Chronic focal glomerulonephritis	43	23/12 after i.v. iron			+
15 Drug (analgesic) induced nephropathy	71	14/12 after i.v. iron		+	
Total (dialysed patients)			1	1	3
Total (patients given iron)			2	1	4
Normals (15)			1	14	0

tion of bone marrow cellularity and its relationship to myeloid-erythroid ratio is the relative size of individual cells. Myeloid precursors are much larger than erythroblasts, and thus a change in their numbers will *have a greater effect on marrow* cellularity So, until quantitative data related to this are available, a full understanding of marrow cellularity and the myeloid-erythroid ratio cannot be attained.

In CRF excess iron stores are usually found in the marrow [9 10] however our group of patients shows a wide range of iron stores. Interestingly *Barry* [11] concluded that in normal livers, slight or moderate siderosis was more common than was believed and was probably not incompatible with normal storage iron levels. Thus, a large range of iron stores in normal marrow may reflect physiological differences in the handling of iron in the normal population. This would also apply to CRF patients and may explain the rise in haemoglobin in patients on dialysis after intravenous iron [12, 13] even though they appeared to have adequate iron stores [12] Another explanation of response to intravenous iron in the presence of apparently adequate stainable iron in the marrow is that much of this iron is not available: this is particularly likely after previous parenteral iron therapy [14]

In aspiration marrow biopsies, only a small proportion of the body's marrow is sampled thus, variations in marrow cellularity throughout the body or metaplasia of fat cells in the marrow cannot be properly assessed. However we have not been able to confirm previous reports of marrow hypocellularity or hypercellularity A normocellular marrow in the presence of anaemia may represent an inability of the marrow to respond to the anaemia, insufficient stimulation of the marrow or both of these. The second seems to be the case in CRF However the slightly higher proportion of erythroblasts in 6 of our cases (lower myeloid-erythroid ratio) must be taken as indicative of increased response. Uraemic bone marrow appears to have erythroblasts capable of normal erythropoietic responses, but that response is incomplete due to low erythropoietic levels [15] The situation may be complicated by the presence of toxic metabolites [1] and a serum factor which inhibits erythropoietin-stimulated erythropoiesis [16]

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D O Ho-Yen,
University Department of Pathology
Haematology Section, Ninewells Hospital,
Dundee, DD1 9SY (Scotland)

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Status of Ascorbic Acid in Iron Deficiency Anaemia and Thalassaemia

Bharati Chatterjea, Arati Maitra D K. Banerjee and A K. Basu

Department of Haematology School f Tropical Medicine, Calcutta, India

Key Words. Ascorbic acid Homozygous thalassaemia Iron Deficiency anaemia
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Abstract. The status of ascorbic acid was studied at the levels of platelet and leucocyte 32 cases of iron deficiency anaemia, 35 cases of thalassaemia and 18 normal subjects. was found that in iron deficiency anaemia, platelet ascorbic acid was significantly higher an normal values indicating tissue excess and came down sharply after treatment with on. In thalassaemia, associated presumably with iron overload, ascorbic acid status of atelets was definitely lower than normal indicating tissue deficiency Leucocytic level ascorbic acid was not as good an index of ascorbic acid status as the platelet level.

Severe iron overload resulting from pro- nged dietary intake or primary disorder of on metabolism or multiple blood transfu- ons, has been shown to produce ascorbic id deficiency at the tissue level [5–7 11 3 14]. This finding raised the question hether iron deficiency was responsible for ny change in the tissue ascorbate level. eucocyte ascorbic acid concentration was levated *in iron deficiency anaemia* and de- reased in iron overload [4] Available liter ture does not furnish any information re arding the platelet concentration of ascor ic acid in iron deficiency anaemia. A study was undertaken to elaborate the status of scorbic acid in iron deficiency anaemia and halassaemia at the tissue level. Ascorbic acid content of platelets and leucocytes were estimated because they are considered as the most sensitive indices of ascorbic acid status of an individual [1 12]

Materials and Methods

Investigations were carried out in total of 85 subjects including normals (18), β-thalassaemia (7), HbE-thalassaemia (28) and iron deficiency anaemia (32). Diagnosis of iron deficiency anaemia was confirmed both by haematological findings such as hypochromia in peripheral blood films, haemoglobin and haematocrit values and biochemical parameters like serum iron (SI), total iron binding capacity (TIBC) and transferrin saturation. In the follow-up study of 10 cases of iron deficiency anaemia, all the parameters were esti-

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University Department of Pathology
Haematology Section, Ninewells Hospital,
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mated before and after treatment. Clinical diagnosis of thalassaemia was confirmed by paper electrophoresis of haemoglobin and estimation of fetal haemoglobin by standard alkali denaturation technique.

Haematological examinations were done by standard techniques. SI and TIBC were measured by the modified method of *Peters et al* [9], ICSH [3], and *Ramsay* [10] respectively. Estimation of ascorbic acid in platelets and leucocytes was done by the method of *Denson and Bowers* [2] as modified by *Attwood et al.* [1]

Separation of platelets from leucocytes was performed as follows: Venous blood collected in siliconised tube in the presence of EDTA was mixed thoroughly with half the volume of 6% dextran and allowed to settle at 37 °C for 1 h. After the sedimentation of the erythrocytes, the upper layer (a mixture of plasma-dextran-leucocytes and platelets) was withdrawn with a siliconised pipette, from this the upper two-third layer (rich in platelets) was separated from the lower one-third layer (rich in leucocytes). Leucocyte and platelet counts were done from a portion of each of the two layers. The leucocyte rich fraction was freed from the remaining erythrocytes by washing with distilled water. Cell pellets of platelets and leucocytes were formed from the two fractions by centrifugation at 3 000 rpm for 30 min. The pellets were finally disrupted in the presence of 6% trichloroacetic acid by repeated freezing and thawing until complete disintegration of the cells took place.

Results

Biochemical evidences of iron deficiency in 32 subjects with IDA are recorded in table I. The platelet and leucocyte level of ascorbic acid in different groups of subjects have been compared in table II. Data representing the different parameters in iron deficiency anaemia before and after therapy have been shown in table III.

In HbE-thalassaemia and β-thalassaemia, the mean values of platelet ascorbic acid were remarkably low being 33.7 and 23.7 μg/10¹⁰ cells, respectively and their difference with the normal mean was statistically significant (table II). In iron deficiency anaemia the mean value (102.3 μg/10¹⁰ cells) of platelet ascorbic acid was significantly higher than the normal mean (49.3 μg/10¹⁰ cells) and the difference was statistically significant. The mean value of leucocytic ascorbic acid in either of the groups was not significantly different from the normal mean. So far as the normal and thalassaemia cases are concerned, present findings are in close agreement with those of other workers [5–7, 11, 13, 14]. Present findings with respect to iron deficiency anaemia agree well with the leucocytic level of ascorbic acid recorded by *Jacobs et al.* [4]. In the normal group of subjects only 3 of 18 had a platelet ascorbate level between 70 and 80 μg whereas the rest had values below 60 μg but, in iron deficiency anaemia, the platelet level of ascorbic acid was below 60 μg in 8 cases and it lay between 60 and 80 μg in 7 cases, between 80 and 100 μg in 4 and between 100 and 270 μg in 13 cases. Thus, in iron deficiency anaemia ascorbic acid content of platelets is markedly elevated in the majority of cases. The leucocytic level of ascorbic acid did not show any significant difference from the normal value.

After the initiation of iron therapy with ferrous sulphate, 10 of the 32 patients were

Table I. Laboratory findings in 32 cases of iron deficiency anaemia

	Range	Mean ± SE
Haemoglobin, g/dl	2.9 – 11.9	7.39 ± 0.4
Haematocrit, ml/dl	10 – 41	24.5 ± 1.4
Serum iron, mg/l	0.2 – 0.5	0.36 ± 0.029
TIBC, mg/l	3.15 – 4.45	3.67 ± 0.49
Transferrin saturation, %	5.7 – 13.8	9.79 ± 0.38

followed up. The period of follow-up study varied from 4 to 22 weeks. Table III reveals that 6 of the 10 cases were severely anaemic and extremely deficient in iron as shown by their low haemoglobin and low transferrin saturation. Following iron therapy the Hb level increased and there was a marked decline in the platelet ascorbic acid level in all the cases. There was a slight decline in the leucocyte ascorbic acid level. It was noticed

Table II. Mean values of platelet and leucocyte ascorbic acid in different groups of subjects

Subjects	Number of cases	Platelet ascorbic acid μg/10[10] cells		Leucocyte ascorbic acid μg/10[8] cells	
		range	mean ± SE	range	mean ± SE
Normal	18	28 – 80	49.3 ± 3.5	2 – 25	10.78 ± 1.6
Iron deficiency anaemia	32	25 – 270	102.3 ± 11.8 t = 3.5 p<0.01 HS	5 – 25	14.0 ± 1.19 t = 1.74 p<0.1 NS
HbE thalassaemia	28	10 – 77	33.7 ± 3.7 t = 2.85 p<0.01 HS	2 – 24	7.5 ± 1.0
β-Thalassaemia	7	9 – 47	23.7 ± 5.3 t = 3.84 p<0.001 HS	4 – 19	7.1 ± 2.1

HS = Highly significant NS = not significant.

Table III. Data showing the different parameters in 10 cases of iron deficiency anaemia before and after treatment

No	Hb, g/dl		Transferrin saturation, %		Platelet ascorbic acid μg/10[10] cells		Leucocyte ascorbic acid, μg/10[8] cells	
	before	after	before	after	before	after	before	after
1	2.9	11.31	5.9	21.5	270	81	26	14
2	3.48	12.17	6.2	35.0	211	79	23	8
3	4.35	10.15	8.5	20.0	118	60	16	10
4	8.12	11.02	11.4	25.4	132	85	18	15
5	6.09	11.6	9.2	28.0	112	79	16	10
6	7.0	12.25	8.0	20.0	84	52	21	20
7	3.8	12.01	8.1	25.0	128	44	14	7
8	2.78	12.9	10.0	27.0	51	35	2	1
9	8.75	11.3	5.7	18.9	139	43	6	5
10	2.8	12.59	6.4	52.9	89	33	22	7

t 4.4 $p < 0.01$ highly significant

during the study that though there was a gradual decline in the ascorbate level throughout the whole period of treatment, the decrease in the platelet level of ascorbic acid was most prominent between the 4th and 5th week of iron therapy. Thereafter the decline was slow. As shown in table III maintenance of high normal values in 4 cases (1, 2, 4, 5) even after the initial decline might in all probability be due to the incomplete repletion of tissue iron store. Table III further shows that the post treatment levels of leucocyte ascorbic acid were not significantly lower than the initial ones. Though the first 2 cases with an extremely high level of platelet ascorbic acid were severely anaemic, no correlation between the ascorbate level and degree of anaemia or transferrin saturation could be found in other cases. The post-treatment values of platelet ascorbic acid were compared with the pre treatment ones and the difference between them was found to be statistically significant.

The present findings further indicate that the platelet level of ascorbic acid is a better index of ascertaining the ascorbic acid status of an individual than leucocyte level.

Discussion

Tissue deficiency of ascorbic acid in thalassaemia as noticed here is comparable with similar conditions of iron overload [5–7, 11, 13, 14]. As an explanation of tissue deficiency of ascorbic acid in iron overload it has been suggested that iron stores, which are in the ferric form and represent a massive oxidative potential, can lead to irreversible oxidation of the available ascorbic acid [14]. Hence tissue excess of ascorbic acid in iron deficiency anaemia with depleted iron stores is presumable. Present observations support this view particularly with respect to the platelet level of ascorbic acid. Elevated leucocytic ascorbic acid in iron deficiency anaemia, as reported by *Jacobs et al.* [4] were, in all probability due to the presence of platelets in the leucocyte fraction studied by them because they did not separate the leucocytes from the platelets and calculated the results from the number of leucocytes only as done by *Denson and Bowers* [2]. Depletion of tissue ascorbic acid following iron therapy might be due to increased catabolic rate as suggested by *Jacobs et al.* [4] or due to its involvement in the reduction of ferric iron of ferritin [8, 12]. The exact pathway of its action remains to be explored. Present findings tend to conclude that in iron deficiency anaemia, due to the shortage of tissue iron, ascorbic acid remains unutilised and its elevation of tissue level occurs. Following iron therapy along with the repletion of tissue iron stores, ascorbic acid is utilised in the transfer of iron from the storage compartment to the metabolic compartments and consequently its tissue level declines.

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Dr D. K. Banerjee,
Assistant Professor of Biochemistry
Department of Haematology
School of Tropical Medicine,
Calcutta, 700 073 (India)

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Red Blood Cell Membrane and Serum Sialic Acid in Relation to Erythrocyte Sedimentation Rate

H. Levinsky, G. Rothman, M. Lapidot and D. Allalouf

Connective Tissue Research Unit, Departments of Internal Medicine A and B, Hasharon Hospital, Petah Tiqva, Israel

Key Words. Erythrocyte sedimentation rate · Red blood cell · Sialic acid

Abstract. Sialic acid was determined in red blood cell (RBC) membrane and in serum, and correlated to erythrocyte sedimentation rate (ESR). For this purpose blood samples were obtained from 57 patients, regardless of their pathological condition, and divided into groups according to the ESR. 15 blood samples obtained from healthy individuals whose ESR was lower than 20 mm/h served as controls. Sialic acid was released from RBC membranes obtained by hemolysis, and from serum glycoproteins precipitated with ethanol by treatment with 0.1 *N* HCl at 80 °C for 1 h. The results showed the sialic acid contents of both membranes and serum to be higher in most of the groups with elevated ESR, as compared to the control group, although no quantitative correlation was noticed. Statistical evaluation showed highly significant differences between the group of pathological samples as a whole and the control group in the sialic acid content of both RBC membranes and sera.

The measurement of the erythrocyte sedimentation rate (ESR) is one of the oldest investigations in clinical medicine. As performed in the clinical laboratory, the sedimentation is a complex phenomenon involving a multitude of factors pertaining to both red blood cells (RBC) and plasma [3, 9]. The Stokes's law (equation 1), which applies to the rate of fall of a single sphere, can be used in this case only with approximation on account of the shape, deformability and the high number of RBC and their capacity to aggregate in the form of rouleaux, the size of which is a critical factor [9, 12, 13].

$$V = 2/9\, r^2 (d_1 - d_2)\, G\, n^{-1} \quad (1)$$

V = velocity of fall; r = radius of particle; d_1 = density of the particle; d_2 = density of the medium; G = gravitational constant; n = viscosity of the medium.

The capacity of the RBC to aggregate depends on the composition of the plasma and on the fact that the cells in suspension normally have a negative charge resulting from the negatively charged sialic acid

groups on the surface of their membrane [7] which causes them to repel each other with a force represented in equation 2. It follows that the net charge contributed by sialic acid can indirectly influence the ESR by regulating the size of the aggregates (r equation 1)

$$F = Q \; Q_2 \; \epsilon^{-1} \; l \qquad (2)$$

F = repelling force Q Q_2 = net charges, ϵ = dielectric coefficient of the medium, l = distance.

The present work was designed to examine whether there exists a relationship between the sialic acid content of RBC membranes and ESR.

On the other hand, as the dielectric coefficient of the plasma increases, the repulsion force between adjacent RBC decreases and it becomes easier for them to aggregate and form rouleaux. The dielectric coefficient depends on the concentration and charge of plasma proteins. Most of the pathological states characterized by elevated ESR are associated with high levels of certain plasma glycoproteins, named acute phase proteins, the carbohydrate units of which terminate in sialic acid [5]. In the present work we examined the relationship between ESR and serum glycoprotein sialic acid, considering that, apart from representing a measure of the level of acute phase proteins, it could be directly responsible for the dielectric properties of the plasma, due to the charge contributed by it to the glycoproteins in solution.

Materials and Methods

Blood samples were obtained from 57 patients suffering from various diseases with ESR above 20 mm/h, and from 15 healthy blood bank donors whose ESR was lower than 20 mm/h. The patients were divided into groups according to the ESR, as seen in figure 1. The anticoagulants used were sodium citrate 3.8% (1:4 parts blood) for the ESR determination by the Westergren method [17], potassium ethylenediaminetetraacetate 2.5 mg/2 ml blood for RBC counting with Coulter S counter and acid citrate dextrose buffer for the preparation of RBC membranes.

Preparation of RBC membranes was carried out according to *Schaner et al.* [10], briefly as follows: suspension of about 10^8 RBC in physiological saline was layered onto Millipore cellulose nitrate filter (pore size 1.2 µm, diameter 47 mm), in a Millipore filtering apparatus (Sterifil filtering system, Millipore Co.). After suction, the RBC were washed twice on the filter with 15 ml 0.3% sodium chloride. Hemolysis was performed by two washings of the filter with 20 ml distilled water.

Sialic acid was determined in RBC membranes [10] after hydrolytic release carried out by incubating the membranes layered upon the filters for 1 h at 80 °C with 0.1 *N* HCl. The hydrolysate was washed once with ethyl ether to remove lipids and passed through column of 0.7 cm diameter containing 6 cm height of Dowex 2 × 8, 100–200 mesh (Sigma), in the formate form. The resin was then washed with 10 ml distilled water and the sialic acid subsequently eluted with 8 ml 2 *N* formic acid. The eluate was evaporated to dryness at 30 °C and sialic acid was determined in the residue by the periodic acid-thiobarbituric acid assay [1, 16] and expressed per 10^9 RBC. The determination of sialic acid in serum glycoproteins precipitated with ethanol was carried out according to *Nieber* [8] and expressed per 100 ml. The statistical evaluation of the differences between groups was done by the Student's t test.

Results

The results of sialic acid determinations in RBC membranes and serum as a function of ESR are presented in figure 1. It can be seen that the content of sialic acid in the membranes from groups with ESR above 60 mm/h was higher than that of the control group. Statistical evaluation, however showed no significant differences between

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normal ESR, and those with normal ESR separately showed significantly higher levels of sialic acid in both RBC membranes and serum in the pathological group (table II).

Discussion

It is clear from equation 2 that the amount of charge present on the erythrocytes membrane surface, assumed to be contributed by the sialic acid residues, is one of the factors determining the repelling force between them and their capacity to form aggregates. This has been also confirmed experimentally by *Stickl and Böcker* [11] who observed an agglutination of RBC treated with neuraminidase when mixed with serum. Although the differences reported by us in membrane sialic acid between the various groups with elevated ESR and the control group were, generally of no statistical significance, the tendency for increase in the pathological group is clear (table II). In fact, increase in sialic acid should result in decreased sizes of RBC aggregates (r, equation 1) and in a lower velocity of sedimentation. These changes, however would be canceled in the presence of an increase in the dielectric coefficient of plasma (ε, equation 2) which is dependent on the concentration and charge of the glycoproteins. Indeed, the levels of some glycoproteins have been reported to be increased in plasma in many pathological conditions characterized by high ESR [2, 5 14 15] and can also be deduced from our findings of elevated concentrations of sialic acid. In this connection, the importance of this residue has been supported by the observation that treatment of serum with neuraminidase resulted in the lack of aggregation of erythrocytes [11] It appears, therefore, that the importance of the RBC component of blood would be rather negligible in determining the ESR and that the critical factors reside in the plasma. This has been convincingly demonstrated in experiments consisting in the mixing of erythrocytes originating from blood samples with either high or low ESR with plasmas from bloods with high or low ESR [11]

The tendency for increase in RBC membranes sialic acid in conditions associated with elevated ESR deserves some comment. Previous investigations have shown that, in these conditions, the liver cells are stimulated by as yet unknown mechanisms to produce increased amounts of several acute phase proteins to which protective roles have been attributed [5] From our results it could be tentatively proposed that the hemopoietic system is also stimulated in the enrichment of the RBC membranes with sialic acid, and, possibly glycoproteins the importance of which in the mentioned conditions remains to be investigated.

The results obtained in serum indicate that the concentrations of sialic acid cannot be correlated to the ESR. This, in our opinion, is not surprising since fibrinogen, which has been reported to play a role in ESR [4], has not been studied in this work and the various conditions with elevated ESR are associated with various types of glycoproteins [6, 14] in which the sialic acid content is known to differ [5] In addition, the contribution of many other factors as plasma viscosity the number size and deformability of erythrocytes as well as the possible involvement of pathology of the liver which is held responsible for the biosynthesis of plasma glycoproteins, in the heterogenous group of patients included in this work has to be taken into consideration.

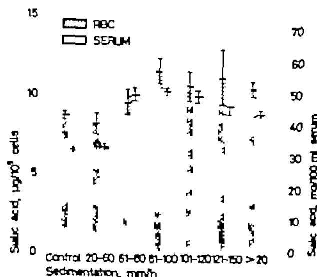


Fig. 1. Sialic acid content of RBC membranes and serum in relation to ESR. The vertical lines above the bars represent standard errors (SE). Sialic acid was determined after hydrolytic release from RBC membranes or from serum glycoproteins in 0.1 N HCl at 80 °C for 1 h.

any group and the control except for the group with ESR of 81–100 mm/h (table I).

Sialic acid in serum was higher in all groups with ESR above 20 mm/h as compared to that with normal ESR, the differences being highly significant beginning from the group with ESR above 60 mm/h (table I). Compilation of all the individual results after dividing the 72 samples examined into two groups, those with higher than

Table I. Sialic acid in RBC membranes and in serum, in relation to ESR

ESR mm/h	Number of samples	Sialic acid					
		RBC, µg/10⁹ cells			Serum, mg/100 ml		
		range	mean ± SE	p	range	mean ± SE	p
<20	15	6.2 – 9.9	8.63 ± 0.23		30 – 37	33.7 ± 1.10	
20 – 60	9	5.9 – 12.0	8.12 ± 0.66	NS	32 – 42	36.0 ± 1.20	NS
61 – 80	10	4.5 – 14.0	9.38 ± 0.82	NS	23 – 70	48.8 ± 4.29	<0.005
81 – 100	17	7.8 – 19.4	11.40 ± 0.84	<0.005	39 – 62	51.6 ± 2.01	<0.001
101 – 120	10	6.6 – 15.7	10.40 ± 0.95	NS	32 – 79	47.7 ± 4.00	<0.005
121 – 150	11	6.3 – 22.5	11.00 ± 1.77	NS	35 – 71	46.8 ± 3.98	<0.005

NS = Nonsignificant.

Table II. Sialic acid in RBC membranes and in serum in patients with elevated ESR

	ESR mm/h	Number of samples	Sialic acid			
			RBC, µg/10⁹ cells		Serum, mg/100 ml	
			mean ± SE	p	mean ± SE	p
Controls	<20	15	8.63 ± 0.23		33.70 ± 1.12	
Elevated	>20	57	10.28 ± 0.44	<0.005	47.10 ± 1.50	<0.001

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H. Levinsky PhD,
Connective Tissue Research Unit,
Hasharon Hospital, Petah-Tiqva (Israel)

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Different Binding Proteins for Folic Acid in Serum of Patients with Acute Hepatitis[1]

R. Corrocher, L.M. Bambara, M.L. Pachor, A.M. Stanzial and D. Biasi

Istituto di Patologia Medica II (Direttore: Prof. *Giorgio D. Sandre*), Università di Padova, Verona, Italia

Key Words. Acute hepatitis · Folate binder · Folic acid

Abstract. Serum from 44 volunteers and 21 patients with acute hepatitis and 18 with cirrhosis were tested for the total and unsaturated folate binding capacity (TFBC-UFBC). A high level of TFBC was noted in acute hepatitis ($p < 0.001$) and in cirrhosis ($p < 0.05$), whereas only in the last case a reduced UFBC was present ($p < 0.05$). When serum from patients with acute hepatitis was eluted on Sephadex G-200 after incubation with radioactive folic acid (500 pg), two main peaks were observed, one in the exclusion peak of protein and the second in the position of the only binder found in normal serum (MW 43,000). A third small peak was also present in the position corresponding to a MW of 20,000. These findings documented the release of binders from hepatic cell when a massive necrosis of liver occurs.

The presence of folate binders inside the cell have been well established in animal and human liver [*Corrocher et al.*, 1974, 1978; *Zamierowski and Wagner*, 1974; *Markkanen et al.*, 1975]. Some of these binders are free in the cytoplasm but others are linked to the mitochondria and nuclei [*Zamierowski and Wagner*, 1977]. Normal serum has a measurable capacity to bind folate which increases in hepatic disorders [*Colman and Herbert*, 1976; *Corrocher et al.*, 1979]. It has therefore been postulated that liver may be one source of serum-binding protein together with leukocytes.

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In a previous elution study of normal serum only one folate-binding protein was detected. However, one may postulate that in the presence of extensive cell necrosis, such as in acute hepatitis, the binders present inside the cell will be released into the blood stream. This study presents evidence that in this condition more than one folate binder appears in serum.

Material and Methods

Serum was obtained from 44 normal volunteers (24 males and 20 females), aged from 18 to 58 years, 21 patients with acute hepatitis (all Australia antigen positive) and 18 patients with hepat-

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H. Levinsky, PhD
Connective Tissue Research Unit,
Hasharon Hospital, Petah-Tiqva (Israel)

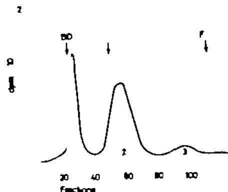


Fig. 2. The elution pattern of serum from patients with acute hepatitis. BD = Bleu dextran, A = albumin; FA = folic acid.

main peaks in both the cases one other small peak was constantly present in the position corresponding to a MW of 20,000–24,000. The first peak eluted closed with the position of the void volume, while the second peak was found later than albumin in a position corresponding to a MW of 43,000 Only this binder was present in normal or cirrhotic serum whereas no peaks were noted when the radioactive folic acid was eluted in the absence of serum. in this case all the radioactivity accumulated in the position of free folic acid (fig. 2).

Discussion

Different binding proteins for folic acid are present in li er cell either free in the cytoplasm or structured in the mitochondria or nuclei [*Corrocher* et al., 1974 *Zamierowsky and Wagner* 1977]. Since TFBC is raised in liver diseases, it has been suggested that liver may be a source of folate binder [*Colman and Herbert* 1976 *Corrocher et al.* 1979]

The findings presented here support this hypothesis. However as in normal serum, only one binder may be detected, whereas in acute hepatitis we have different binders, it means that only in the presence of a massive cell necrosis some of the binders (normally structured) may be released into the circulation and detected with our method. The TFBC was higher than normal in cirrhosis and even higher in acute hepatitis ($p < 0.05$ and $p < 0.001$ respectively). The saturation of serum was 78 4 / in cirrhosis and 85°/ in acute hepatitis against 84 4 / in normal. These findings are relatively unexpected in fact the serum in acute hepatitis was more saturated than serum in cirrhosis in spite of a higher TFBC (276 vs. 231 pg/ml). This finding may be partially explained with the presence in serum of patients with acute hepatitis of different binders as documented in figure 2. If these binders had a different affinity for radioactive folic acid, the total and unsaturated binding capacity of this serum might not be compared with that found in normal or cirrhosis, being the net result of different affinities in acute hepatitis. A further indirect evidence for this hypothesis is represented by the fact that in cirrhosis, TFBC was not statistically different from that in acute hepatitis in spite of a large necrosis of liver documented in these patients by the high level of GOT the method for measuring TFBC and UFBC in acute hepatitis might not be suitable in the presence of different binders.

The characteristic pattern of folate-binding proteins found in acute hepatitis differed from that noted in normal and cirrhotic serum [*Colman and Herbert* 1976 *Corrocher et al.* 1979] and represents evidence that in the presence of cell necrosis the intracellular binders may be released into the circulation similar binders were observed in the cyto-

ic cirrhosis. The serum was separated from clotted blood within 2 h and stored at −20 C for not more than 15 days. All the procedures were performed using disposable gloves and mask to prevent virus infection.

Determination of Total and Unsaturated Folate Binding Capacity. This was performed by the method recently described but slightly modified [*Corrocher* et al., 1979]. The method is based on the displacement at low pH (0.0285 *M* of citric acid, monopotassium phosphate, barbital and boric acid) of endogenous folate which is then removed with albumin-coated charcoal (ACC). The binder is then ready to bind exogenous-labelled folate. The modification introduced here concerned the preparation of the 'zero control tube': since normal serum contains about 3.0 g/dl of albumin (i.e. 12.0 mg/0.4 ml of serum), an equal amount of human albumin was added to the 'zero tube'. This fact minimizes the possible interference of serum albumin (nonspecific binder for folic acid) in the quantification of binding capacity.

Chromatography. 5 ml of serum from two different patients with acute hepatitis (R. C., A. C.) were treated with the acidic solution (Vo/Vo) used for TFBC determination and then absorbed with 3 ml of ACC to remove endogenous folate. When albumin completely saturates the charcoal, ACC will remove only free folate: the proportion between serum and ACC used in the experiment was chosen since 3 ml of ACC removed 95% 10 ng of labelled folate added to 5 ml phosphate-buffered saline plus acidic solution. In this condition, as traces of free folat remained in the supernatant, the possibility that ACC removed the binder is negligible. The pH was restored to 7.5 with sodium hydrate before adding exogenous radioactive folic acid (500 pg). After 30 min of incubation at 37 °C, the free radioactivity was removed with ACC by centrifugation (10 min at 3,000 rpm). 5 ml of radioactive supernatant was eluted from a Sephadex G 200 column (2 × 60 cm) equilibrated with phosphate-buffered saline (pH 7.4) and calibrated with Bleu dextran (BD), albumin (A) and free folic acid (FA). The recovery of radioactive folate from the eluate was measured by integrating the area of radioactive peaks: it was 96–98% when compared with the radioactivity of 500 pg of labelled folate eluated in phosphate-buffered saline in absence of serum (binder). 100 2-ml fractions were collected and the radioactivity of 1.0 ml of each fraction was measured in 10 ml of Instagel (Packard) and counted using a liquid scintillation counter (Packard). The values, corrected for quenching with an external standard method, were plotted against the fraction number.

Results

Figure 1 shows the findings of TFBC and UFBC obtained in our patients. Mean normal TFBC was 160 ± 62 pg/ml (range 89–282 pg/ml). Mean normal UFBC was 25 pg/ml (range 3.5–56 pg/ml). The saturation of normal serum was 84%. All patients with acute hepatitis were in the acute phase of disease: serum glutamic oxaloacetic transaminase (GOT) ranged from 1,700 to 3,200 U/l. In these patients TFBC varied from 198 to 509 pg/ml: mean value 276 ± 91 pg/ml ($p < 0.001$). The mean UFBC was 42 ± 28 pg/ml (not significant). In cirrhosis the mean TFBC was 231 ± 117 pg/ml ($p < 0.05$) and UFBC 50.0 ± 31.6 pg/ml ($p < 0.05$) (fig. 1). The transaminase GOT ranged from 24 to 197 U/l in this group of patients.

Chromatographic study of hepatic serum showed a radioactivity profile with two

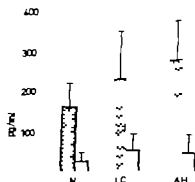


Fig. 1. Mean levels (± SD) of TFBC (stippled) and UFBC (open) in normals (N) and in patients with liver cirrhosis (LC) and acute hepatitis (AH).

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Associated Red Cell Enzyme Deficiencies and Their Significance in a Case of Congenital Enzymopenic Methemoglobinemia

A. Das Gupta, Mangal S. Vaidya, J. P. Bapat, Roshan S. Pavri, A. J. Baxi and S. H. Advani

Blood Group Reference Centre (Indian Council of Medical Research), Seth G. S. Medical College, and Tata Memorial Hospital, Parel, Bombay

Key Words. Congenital methemoglobinemia · Glutathione peroxidase · Glutathione reductase · NADH-methemoglobin reductase · Pseudomosaicism

Abstract. Examination of the red cell enzyme profile in a case of congenital methemoglobinemia has shown associated deficiencies of glutathione reductase (GR) and glutathione peroxidase (GSHPx) in addition to NADH methemoglobin reductase deficiency. Contrary to expectations, GR and GSHPx deficiencies do not seem to have contributed to the methemoglobinemia in this case. The lack of symptoms in spite of a high methemoglobin (Hi) level (35%) appears to be due to the restriction of Hi to a small percentage of red cells.

Introduction

Methemoglobinemia can be defined as a clinical condition in which more than 1% of hemoglobin (Hb) in circulation is oxidized to methemoglobin (Hi) [1]. The defect can either be hereditary or acquired in origin. Deficiency of enzyme-reduced nicotinamide adenine dinucleotide methemoglobin reductase (NADH-MR) [1] and structural abnormalities of the globin chains like HbMs [2] are examples of hereditary methemoglobinemia. Deficiencies of reduced nicotinamide adenine dinucleotide phosphate methemoglobin reductase (NADPH-MR) [3], ascorbic acid [1] and reduced glutathione (GSH) [4] which play a minor role in reduction of Hi *in vivo* are not known to be associated with methemoglobinemia. Many of the household and industrial chemical compounds and therapeutic agents (especially aniline derivatives, nitrites or nitrates), and their breakdown products, if present in the circulation in sufficient amounts, can cause methemoglobinemia in otherwise healthy individuals [5–7]. The high oxygen affinity of Hi and the consequent tissue hypoxia is responsible for most of the symptoms and signs of methemoglobinemia [1].

We present a case of congenital methemoglobinemia due to NADH-MR deficiency in an Indian Muslim. The rarity of this condition and the relatively less morbidity in this case, in spite of two other associated red cell enzyme deficiencies, have prompted us to report it.

plasm of liver cell [*Zamierowski and Wagner 1977*]

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Dr Roberto Corrocher
Istituto di Patologia Medica II,
Policlinico di Borgo Roma,
I-37134 Verona (Italy)

20–30% reduction in GR activity has been noted in the red cells in many cases of NADH-MR deficiency [24]. The exact cause for this association is not known. In some cases this could be due to a dietary deficiency of riboflavin [1, 23, 25]. Similarly a dietary deficiency of selenium is known to reduce the GSH Px activity [26]. A genetically determined reduction in GSH Px activity has also been reported recently in the Jews and the Mediterranean populations [26]. In absence of family studies it is not possible to determine which of these factors is responsible for the low GR and GSH Px activities in our case.

That deficiencies of GR and GSH Px are not functionally significant in this case is apparent from the Hi level (35%), which is consistent with the classical homozygous state for NADH-MR deficiency [1, 23]. One would have expected a much higher Hi level if these enzyme deficiencies had contributed to the methemoglobinemia. Absence of a significant shift in the oxygen dissociation curve (done earlier) further supports this view. Instability of red cell GSH is the outcome of the same oxidative stress which causes Hi formation. This is because protection of Hb and GSH against oxidation are interdependent phenomena [27]. The low GSH stability in this case is, therefore, not unexpected. In a recent paper *Beutler* [26] questioned the role of GR and GSHPx deficiencies in the genesis of nonspherocytic hemolytic anemia and labelled these enzyme deficiencies as 'nondiseases'. The same appears true for their role in methemoglobinemia as well.

The relative lack of symptoms in our case even in face of a high Hi level is consistent with what has already been described in hereditary methemoglobinemia due to NADH-MR deficiency [1]. As suggested by *Keitt et al.* [28] in 1966 the restriction of Hi to a very small population of red cells (fig. 1), which presumably are older in age ('pseudomosaicism'), could explain this phenomenon. It is possible that the majority of red cells, which are younger and contain very little Hi, function normally. The reserve reducing activity in these cells already referred to, is reported to be competent enough to compensate for NADH-MR deficiency [28].

In absence of data on the Hi and NADH-MR levels in the patient's relatives, it is not possible to be certain about the mode of inheritence of this defect in this patient. The clinical features and laboratory findings, however, favor a homozygous state. History of consanguinity in the patient's parents further strengthens such a possibility since this defect has been found to be prevalent in inbred populations [24].

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Case Report

A. H., a 32 year-old Muslim male from Calcutta complained of easy fatigability and occasional headache of long duration accompanied by slate-gray discoloration of the lips and nails. He was operated upon in 1977 for duodenal ulcer. 1 of his 3 siblings, his elder brother has similar complaints. The other 2 are sisters, and not much information could be obtained about them. The patient's parents are first cousins. None of his relatives could be investigated.

The patient was well built and nourished. He had dusky cyanosis and mild clubbing of fingers which could not be explained because all other systems were found to be normal on examination. There was no neurologic deficit. Investigations were carried out using standard techniques [8] unless otherwise indicated. In June, 1979 his Hb was 15.05 g/dl (normal values (N) = 13–18) packed cell volume = 0.44 l/l (N = 0.40–0.54) mean corpuscular Hb concentration = 34.2 g/dl (N = 31–35) reticulocytes = 0.8% (N < 2) osmotic fragility (in 0.4% sodium chloride) = 52.7% (N = 50–90) *Hi* = 35.03% (N < 1) Hb_f = 0.58% (N < 2) [9] Hb A = 3.0% (N = 0.7–3.0) [10] Hb electrophoresis on paper in phosphate buffer at pH 7 [11] did not show HbM band. Fluorescent spot test for NADH MR deficiency was positive [12]. Elution test for Hi showed it to be restricted to a very small percentage of red cells (fig. 1) [13]. Peripheral smear did not show any morphologic abnormality. No Heinz bodies could be demonstrated either by methyl violet or brilliant cresyl blue staining. Oxygen dissociation curve (done in 1977) is reported to have shown no significant shift to left.



Fig. 1. 'Pseudomosaicism' in the patient's red cells after elution of methemoglobin (Hi) by 0.2 *M* citric acid. The smear has been counterstained with Mayer's hemalum and 0.5% eosin. The small number of ghost cells represent the Hi-containing cells *in vivo*. Also note some intermediately stained cells. × 400

His GSH level was 51 mg%/ RBC (N = 46–101) [14] *GSH stability* = 45.6% (N = 71–101) [15]; *GSH peroxidase* (GSH Px) = 17.5 U/g Hb (N = 33–65.5) [16] *glutathione reductase* (GR) = 4.16 U/g Hb (N = 6.5–8.3) [17] *NADH MR* = 15 U (N = 64–126) [18] NADPH MR = 2.11 U (N = 2–3.5) [19] Catalase = 7.0 U (N = 3.0–6.7) [20] glucose-6-phosphate dehydrogenase = 2.6 U/ml RBC/min (N = 2.8–5.6) [21] and 2,3-diphosphoglycerate = 17.3 μM/g Hb (N = 10.9–19.2) [22]

Discussion

The normal level of Hi in blood (< 1%) reflects the subtle balance between two opposing forces *in vivo*, namely those responsible for its formation and those governing its reduction to Hb. Approximately 95% of the latter activity is mediated through NADH MR which uses NADH as a coenzyme [23]. Rest of this activity resides in NADPH MR, ascorbic acid and GSH, as already mentioned. GR, by virtue of its role in maintaining an optimal GSH level can also affect this balance. Deficiency of GR would lead to reduced GSH level with consequent decrease in Hi-reducing activity. Similarly, a deficiency of GSH Px, would theoretically favor excess Hi formation through the unopposed action of H_2O_2 on Hb molecules.

Interestingly, our patient is deficient not only in NADH-MR, but also shows reduced activities of both GR and GSH Px. A

20–30% reduction in GR activity has been noted in the red cells in many cases of NADH MR deficiency [24]. The exact cause for this association is not known. In some cases this could be due to a dietary deficiency of riboflavin [1, 23, 25]. Similarly a dietary deficiency of selenium is known to reduce the GSH Px activity [26]. A genetically determined reduction in GSH Px activity has also been reported recently in the Jews and the Mediterranean populations [26]. In absence of family studies it is not possible to determine which of these factors is responsible for the low GR and GSH Px activities in our case.

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20–30% reduction in GR activity has been noted in the red cells in many cases of NADH-MR deficiency [24]. The exact cause for this association is not known. In some cases this could be due to a dietary deficiency of riboflavin [1, 23, 25]. Similarly a dietary deficiency of selenium is known to reduce the GSH Px activity [26]. A genetically determined reduction in GSH Px activity has also been reported recently in the Jews and the Mediterranean populations [26]. In absence of family studies it is not possible to determine which of these factors is responsible for the low GR and GSH Px activities in our case.

That deficiencies of GR and GSH Px are not functionally significant in this case, is apparent from the Hi level (35 /), which is consistent with the classical homozygous state for NADH-MR deficiency [1, 23]. One would have expected a much higher Hi level if these enzyme deficiencies had contributed to the methemoglobinemia. Absence of a significant shift in the oxygen dissociation curve (done earlier) further supports this view. Instability of red cell GSH is the outcome of the same oxidative stress which causes Hi formation. This is because protection of Hb and GSH against oxidation are interdependent phenomena [27]. The low GSH stability in this case is, therefore, not unexpected. In a recent paper *Beutler* [26] questioned the role of GR and GSHPx deficiencies in the genesis of nonspherocytic hemolytic anemia and labelled these enzyme deficiencies as 'nondiseases'. The same appears true for their role in methemoglobinemia as well.

The relative lack of symptoms in our case even in face of a high Hi level is consistent with what has already been described in hereditary methemoglobinemia due to NADH MR deficiency [1]. As suggested by *Keitt et al.* [28] in 1966 the restriction of Hi to a very small population of red cells (fig. 1), which presumably are older in age ('pseudomosaicism'), could explain this phenomenon. It is possible that the majority of red cells, which are younger and contain very little Hi, function normally. The reserve reducing activity in these cells already referred to is reported to be competent enough to compensate for NADH-MR deficiency [28].

In absence of data on the Hi and NADH-MR levels in the patient's relatives, it is not possible to be certain about the mode of inheritence of this defect in this patient. The clinical features and laboratory findings, however, favor a homozygous state. History of consanguinity in the patient's parents further strengthens such a possibility since this defect has been found to be prevalent in inbred populations [24].

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Dr A. J Baxi, Assistant Director Blood Group Reference Centre Seth G S Medical College, Parel, Bombay 400 012 (India)

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Effect of Intravenous Adrenalin Infusion and Corticosteroid Treatment in Patients with von Willebrand s Disease

Şinasi Özsoylu Kadir Tuğcu, Işin Cötelli Hikmet Kavcioğlu Medine Urkmez and Türkiz Gürsel

Hacettepe University Faculty of Medicine, Department of Pediatrics, Hematology Section, Ankara, Turkey

Key Words. von Willebrand's Disease Adrenalin Corticosteroid

Abstract. Intravenous adrenalin infusion (5 μg/kg, at most 160 μg in 10 min) normalized partial thromboplastin time (PTT) and ristocetin induced platelet aggregation (RIPA) in 4 of the 14 patients with von Willebrand's disease (VWD) in a short period of time. Although mean factor VIII (F VIII) procoagulant activity was almost doubled 5 min following infusion, this was mainly observed in patients with relatively high baseline (> 2%) AHF activity Mean F VIII procoagulant activity rose by more than 100% following 10 days of corticosteroid treatment (deltacortil 2 mg/kg day at most 60 mg/day). PTT became normal in 6 of the 11 patients, but RIPA normalized only in 2. The improvement of RIPA did not correspond to bleeding time in every patient. These results may suggest that if the baseline AHF activity is relatively high (> 9.5%), corticosteroid could be tried before schedule surgical intervention in patients with VWD

von Willebrand's disease (VWD) is not a rare hereditary bleeding disorder and it occurs in both sexes. In Hacettepe Children's Hospital 279 patients with hemophilia A, 64 with hemophilia B and 35 with VWD were observed between 1963 and 1974 [1] The diagnosis was verified by the prolonged bleeding time, decreased factor VIII procoagulant activity and decreased platelet adhesiveness.

Since the effect of corticosteroid and adrenaline on factor VIII (F-VIII) procoagulant activity in hemophilia A patients and blood donors had been shown by us [2, 3] their effect was evaluated in patients with VWD

Materials and Methods

22 patients with VWD who (or whose parents) accepted this evaluation were the subjects of this study The ages of the patients ranged from 3 to 30 years, 11 were male and 11 female. Bleeding times (BT) were determined by the method of *Ivy et al* [4], one stage prothrombin times (PT), partial thromboplastin times (PTT), thromboplastin generation tests (TGT) and F VIII procoagulant activities were performed according to *Quick* [5], *Rodman* et al. [6], *Biggs and Douglas* [7] and

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Dr A. J Baxi, Assistant Director Blood Group Reference Centre, Seth G S. Medical College, Parel, Bombay 400 01 (India)

begin with (table I) Although mean F-VIII activity was almost doubled at 5 min, this was observed only in those 5 patients with relatively high baseline activity (> 2%). Mean F VIII procoagulant activity of this group was 7.31 ± 3.57% which increased to 13.99 ± 12.83% (91 4% elevation) but it was not found statistically significant ($p > 0.05$). Improvement of the tests was observed in the latter group except in one in whom only bleeding time became normal (table I). The duration of F-VIII procoagulant activity elevation was very short since it was not present in samples obtained at 60 min.

Following corticosteroid treatment for 10 days, AHF activity rose by more than 100% in all cases, the mean baseline activity of 11 patients was 7.63 / which increased to 16.11 / (111 / elevation, $p < 0.01$) PTTs normalized in 6 of 11 patients. With one exception, baseline AHF activity of these 6 cases was relatively high (≥ 9.5%). RIPA became normal in two but bleeding time was normalized in one only (table II).

Table II. Prednisone effect on F VIII procoagulant activity in patients with VWD

Before %	After %	Increment	Normalized parameters
1	2.07	107	PTT
12.95	27 28	110 7	PTT RIPA, BT
16 8	32.8	95 2	PTT RIPA BT stayed prolonged
22.9	45.2	97 4	PTT
9 5	27.5	189 5	PTT
1	2 6	160	
10 65	25	134 7	PTT
2.8	3 6	28 6	
3 3	5 6	69 7	
1.8	2.6	44 4	
1.25	3	140	
X̄ 7.63	16.11	111 14	
SD = ± 6 97	15 68		

Discussion

It has been shown by *Ingram* [10] and confirmed by *Egeberg* [11] and *Gardikas* et al. [12] that intravenous adrenaline infusion increases plasma AHF activity considerably Elevation of AHF activity has also been shown during pregnancy [13 14] following physical exercise [11 15] corticosteroid treatment [2] and pneumoencephalography [16] Very recently it was shown by us that more than 100% elevation of AHF activity occurs in patients with marked iron deficiency anemia [17]

The elevation of F-VIII procoagulant activity in patients with hemophilia and VWD has also been reported following the administration of corticosteroid [2] adrenalin [1] 1-deamino-8-arginine vasopressin [18] pneumoencephalography [16] during hepatitis [19] and pregnancy [13]

Eyster et al. [19] suggested using the F VIII response to adrenaline administration to distinguish certain patients with mild hemophilia from those with VWD since they observed elevation of AHF activity only in patients with VWD by subcutaneous adrenaline administration By intravenous administration, we have previously [1] shown its effect on AHF levels in patients with hemophilia A at the same dose used in present study Though not significant, the elevation of AHF activity was also shown in patients with VWD at 5 min in this study Actually elevation occurred in those patients whose initial AHF activity was more than 2% a. 5 min after adrenaline infusion, PTT normalized in 4 patients in whom

McMillan et al. [8], respectively Ristocetin-induced platelet aggregation (RIPA) was studied as described by *Packman and Mustard* [9] After blood was obtained, 5 µg/kg adrenaline (at most 160 µg) in 10 ml 5% glucose was given intravenously in 10 min and coagulation studies, mentioned above, were repeated at 5 and 60 min from completion of infusion in 14 patients.

Following baseline coagulation studies, corticosteroid (deltacortril 2 mg/kg/day at most 60 mg/day) was given orally for 10 days duration in 11 patients and the coagulation studies were repeated 3 patients, a brother and sister and another girl were given both adrenaline and corticosteroid at different times.

Results

PT of all patients was found not to differ from that of the controls but the nonactivated PTT was prolonged and the TGT indicated decreased F VIII procoagulant activity in all patients. With two exceptions BT was prolonged in all patients. A sister of a boy with normal bleeding time had all the criteria of VWD including abnormal RIPA. The boy's RIPA was 44%, which was almost low normal. Since his sister had the disease, he too was included in the VWD group Because of abnormal RIPA, a girl with normal bleeding time was diagnosed as a VWD patient. F VIII procoagulant activity ranged between 0.5 and 10 8% and its deficiency was very marked (≤ 2%) in 13 of the patients.

No significant changes were observed in PT following adrenaline infusion or corticosteroid treatment. PTT and RIPA became normal following adrenaline infusion at 5 and/or 60 min in 4 of the patients. BT became normal in one improved in another at 5 and/or 60 min and was normal in one to

Table I. Adrenaline effect on F-VIII procoagulant activity in patients with VWD

Before, %	After 5 min, %	After 60 min, %	Remarks
0 89	0.85	0.95	
0.96	0.99	0.89	
1.08	1 19	0.97	
1.[illegible]	1 86	1.25	
0.5	0.5	0.5	
0 6	0 85	0.5	
2.2	4	1 74	
2	1 46	1.2	
0.79	0.85	0.75	
9.5	15.9	11.5	PTT and RIPA normalized at 5 and 60 min and shortest at 5 min
0 7	0.74	0.7	BT normalized at 5 and 60 min
10 8	13.37	12	PTT and RIPA normalized at 5 and 60 min respectively BT was normal to begin with
5.04	10 7	5 61	RIPA improved considerably at 5 min and PTT normalized at 60 min BT improved
9	26	7	PTT and RIPA almost normal at 5 min
X̄ = 3.23 SD ± 3 74	5 66 ± 7 81	3.25 ± 4.1	

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Şinasi Ozsoylu, MD,
From Hacettepe University Faculty of Medicine
Department of Pediatric,
Hematology Section Ankara (Turkey)

AHF activity increased to more than 10%, with correction of RIPA but bleeding time was effected only in 2 of them 5 and 60 min after adrenaline infusion the bleeding time of another patient became normal without considerable AHF level and RIPA changes. In general, no parallelism was shown between the normalization of RIPA and bleeding time following adrenaline infusion. The adrenaline effect on the AHF level was of a very short duration. Therefore, its effect should be explained by the secretion of AHF from storage organs.

PTT became normal in 6 of the 11 patients following corticosteroid treatment in 5 of those patients, the AHF activity increased to over 25% of normal However RIPA normalized in only 2 of them and bleeding time in only one Although RIPA normalized in 2 patients whose AHF activity was more than 25%, it was not improved in all of the patients whose AHF activity rose above this level. Spontaneous normalisation of BT in some occasions has been reported in patients with VWD Therefore, we do not think that occasional improvement of BT should be related to the treatment. By chance the baseline AHF activities were higher in the corticosteroid-treated patients than in the adrenaline-infused group There fore their response to these drugs should not be compared.

In conclusion, this study showed that the elevation of AHF activity due to adrenaline administration or following corticosteroid treatment did not improve RIPA or bleeding time in all the patients, but were, in general, effective in those patients whose AHF activity was relatively high to begin with (mild VWD) Although improvement of RIPA and bleeding time are related to each other they do not correlate on every occasion in this study But our results suggest that corticosteroid treatment could be used in those patients with mild VWD in whom baseline AHF activity is over 10%, before scheduled interventions. Adrenaline administration could also be tried in mild VWD patients in controlling mild bleedings around nonvital organs.

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Şinasi Ozsoylu, MD,
From Hacettepe University Faculty of Medicine
Department of Pediatric,
Hematology Section Ankara (Turkey)

Short Communication

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Sea-Blue Histiocytes in Idiopathic Thrombocytopenic Purpura

The First Report from the Indian Subcontinent

M I Quadri S Majumdar Y N Mehra G Garewal and K C Das

Departments of Haematology Experimental Medicine and ENT Postgraduate Institute of Medical Education and Research, Chandigarh, India

Key Words. ITP Sea blue histiocytes Syndrome of sea-blue histiocytosis

Abstract. Sea-blue histiocytes are described in the bone marrow of a 15-year-old male patient suffering from idiopathic thrombocytopenic purpura. Cytochemically the storage material in these cells appeared to be glycophospholipid. Lipid analysis of blood leucocytes and nucleated cells of bone marrow showed a significant increase in the phospholipids triglycerides and cholesterol as compared to age-matched controls. Serum lipid analysis showed a mild increase of phospholipids as compared to age matched controls.

Introduction

The *syndrome of sea-blue histiocytosis* [5 6] has been reported to exist in two forms, a primary variety which is probably a homozygous state for an as yet undefined enzyme deficiency secondly an acquired variety which is probably a heterozygous state, and is precipitated by some stress which includes both haematological and non haematological disorders. Sea-blue histiocytes appeared to have been described in bone marrows and/or spleens of only 4 patients with idiopathic thrombocytopenic purpura (ITP) [1 4] *but none from the Indian subcontinent.* We here describe sea blue histiocytes in the bone marrow of a 15-year-old male patient with ITP who is of Indo-Aryan origin

Material and Methods

K. S., a *15-year-old male, admitted to the hospital* for severe epistaxis and purpura, was diagnosed as having ITP on the basis of the typical clinical and haematological findings. The platelet count at admission was $12 \times 10^9/l$. In addition, the bone marrow smears showed occasional histiocytes which were 30–60 μm in diameter with an eccentric nucleus and easily discernible nucleolus. Cytoplasm contained coarse granules which stained sea blue to azur blue with Leishman's stain. The granules were periodic acid-Schiff (PAS)-positive, both before and after diastase treatment. Sudan B-positive and negative for iron stain. These cells did not show any autofluorescence. The serum lipid profile assayed in the acute stage showed normal pattern except for mild elevation of phospholipids (patient 294.8 mg/dl control 190 ± 20 mg/dl). The phospholipid fractions were not significantly different from age-matched normal controls. The patient's urine-

Table I. Lipid analysis of leucocytes and nucleated bone marrow cells

	Leucocytes mg/ml packed cells				Bone marrow mg/g cells			
	patient	control (3)			patient	control (3)		
		mean	range			mean	range	
Phospholipid	7.27	3 404	2.12	3.55	5 731	0.770	0.31	1.231
Triglycerides	20.454	6 057	2 16	13.636	13.702	1.903	1 108	3 199
Cholesterol	5 68	2.221	1 4	3 725	3.6487	0 517	0 308	0.921

showed traces of acid mucopolysaccharides by paper chromatography Liver function tests were normal.

The patient had to be put on prednisolone (60 mg/day) on the 10th day after admission Bleeding subsided and the platelet count went on increasing, and on the 20th day of steroid therapy his platelet count was 80 × 10⁹/L At this stage, his leucocytes and nucleated bone marrow cells, removed by differential centrifugation (buffy coat) ere subjected to lipid analysis. Leucocytes from 3 normal age-matched males were taken as control Nucleated bone marrow cells from 3 subjects were taken for control, 1 was 5-year-old male with ITP who had never received steroids. The second case was 35-year-old female with ITP ho had been taking 5 mg prednisolone daily but irregularly for 2 years The third case was 13-year-old male having tropical splenomegaly with anaemia. The results are given in table I His father ho was the only available member of the family was clinically and haematologically normal, but his serum lipid analysis showed moderate elevations of total lipids, esterified cholesterol, triglycerides and phospholipids as compared normal age-matched controls Phospholipid fractionation showed normal sphingomyelin and lecithin ratio

Discussion

The sea-blue histiocytes demonstrated in the bone marrow of the case under discussion appeared to contain glycophospholipid rather than ceroid. The cells were morphologically and histochemically different from those of foamy histiocytes described in spleens from cases with ITP and we have not observed sea-blue histiocytes in any bone marrow of a series of more than 50 cases of ITP studied by us during the last 10 years. It is probable that the presence of sea-blue histiocytes is related to an additional pathogenetic factor(s) operating in our present case.

The relation between sea-blue histiocytosis and abnormalities of lipid metabolism is unclear Normal, subnormal or elevated concentrations of total lipid, cholesterol and phospholipids have been reported [5] It is generally held that the heterozygotes usually do not show any abnormal lipid metabolism [5 6] The serum lipid profile of our patient during the acute phase of the disease did show increased levels of phospholipid as compared to normal age-matched controls. However the ratio of sphingomyelin to lecithin was normal. Abnormalities of lipid content of liver spleen and bone marrow homogenates in the form of increased concentration of total lipids, phospholipids and sphingomyelin with the reversal of sphingomyelin and lecithin ratio, have been reported in the primary form and occasionally in

Short Communication

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Sea-Blue Histiocytes in Idiopathic Thrombocytopenic Purpura

The First Report from the Indian Subcontinent

M I Quadri S Majumdar Y N Mehra G Garewal and K C Das

Departments of Haematology Experimental Medicine and ENT Postgraduate Institute of Medical Education and Research, Chandigarh, India

Key Words. ITP Sea blue histiocytes Syndrome of sea blue histiocytosis

Abstract. Sea blue histiocytes are described in the bone marrow of a 15 year-old male patient suffering from idiopathic thrombocytopenic purpura. Cytochemically the storage material in these cells appeared to be glycophospholipid Lipid analysis of blood leucocytes and nucleated cells of bone marrow showed a significant increase in the phospholipids, triglycerides and cholesterol as compared to age matched controls. Serum lipid analysis showed a mild increase of phospholipids as compared to age-matched controls.

Introduction

The syndrome of sea-blue histiocytosis [5 6] has been reported to exist in two forms, a primary variety which is probably a homozygous state for an as yet undefined enzyme deficiency secondly an acquired' variety which is probably a heterozygous state, and is precipitated by some stress which includes both haematological and non-haematological disorders. Sea-blue histiocytes appeared to have been described in bone marrows and/or spleens of only 4 patients with idiopathic thrombocytopenic purpura (ITP) [1 4] but none from the Indian subcontinent. We here describe sea-blue histiocytes in the bone marrow of a 15 year-old male patient with ITP who is of Indo-Aryan origin

Material and Methods

K. S a 15-year-old male, admitted to the hospital for severe epistaxis and purpura, was diagnosed as having ITP on the basis of the typical clinical and haematological findings. The platelet count at admission was 12×10^9/L In addition, the bone marrow smears showed occasional histiocytes which were 30–60 m in diameter with an eccentric nucleus and easily discernible nucleolus. Cytoplasm contained coarse granules which stained sea blue to azur blue with Leishman's stain. The gran les were periodic acid-Schiff (PAS)-positive, both before and after diastase treatment. Sudan B-positi e and negative for iron stain. These cell did not show any autofluorescence. The serum lipid profile assayed in the acute stag showed a normal pattern xcept for a mild elevation of phospholipids (patient 294 8 mg/dl, control 190 ± 20 mg/dl) The phospholipid fractions were not significantly different from age-matched normal controls. The patient s urine-

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Ferrokinetic Studies and Prognosis in Aplastic Anaemia

D M McCarthy D G Oscier and S M Lewis

Department of Haematology Royal Postgraduate Medical School, London, UK

Key Words. Aplastic anaemia Ferrokinetics Prognosis in aplastic anaemia

Abstract. In a study of 16 patients with aplastic anaemia, an attempt has been made to relate ferrokinetic data to haematological measurements and clinical course There was a positive relationship of cellularity on trephine biopsy with plasma iron clearance and erythrocyte iron turnover but not with red cell utilisation. Bone marrow aspirate provided less reliable information on erythropoietic function than did trephine biopsy There was good correlation between all the ferrokinetic parameters and reticulocyte count, but not between reticulocyte count and marrow cellularity even by trephine. Patients whose ferrokinetic studies indicate a less severe erythroid depression (i.e., plasma clearance T < 200 min and red cell utilisation $> 35\%$) are more likely to survive without transplantation than those with more abnormal ferrokinetic results but such studies alone are of only limited value in clinical management of the individual patient.

Introduction

In aplastic anaemia the need to select patients for treatment by bone marrow transplantation makes it important to have reliable criteria for prediction of prognosis in individual cases. The triad of thrombocytopenia, neutropenia and reticulocytopenia has been used to distinguish patients with a poor prognosis [9] who are likely to die within 4 months of their first clinic visit. The data of *Lohrmann* et al. [8] and *Hellriegel* et al. [5] have suggested that the reticulocyte count rather than the neutrophils and platelets indicates the prognosis. However others have considered the reticulocyte count to be less reliable, especially in patients with infection [4 7] Bone marrow cellularity in biopsy specimens does not correlate with prognosis [3 13] and the occurrence of patchy cellularity in even severe cases may give misleading information in random sampling.

A correlation has been demonstrated between ferrokinetic data obtained with ^{59}Fe and bone marrow cellularity [2, 10 12] but in the hypoplastic range, the data presented by *Najean* et al. [10] showed that the

the acquired form of the disease [5 6] The homogenate of nucleated cells from the bone marrow of our patient also showed increased concentrations of the major lipid fractions including phospholipids The leucocyte lipid analysis of our patient revealed increased concentrations of phospholipids, triglycerides and cholesterol as compared to the normal age and sex matched controls. The relevance of lipid abnormalities to the appearance of sea-blue histiocytes remains as yet unclear

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Dr K. C Das, Professor and Head,
Department of Haematology Postgraduate Institute of Medical Education and Research,
Chandigarh-160012 (India)

Erythroid cellularity

1 Not more than two erythroblasts seen in the fragment trail

2 Scattered areas of erythroblasts

3 Active erythropoiesis in the presence of general hypocellularity

The grading of all samples was agreed after independent assessment by two observers.

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For eticulocyte counts, smears were made from blood after incubation for 20 min at 37 °C ith New methylene blue. Counts were taken as the mean of measurements on five samples around the time of the ferrokinetic study

Table II. Ferrokinetic data related to cellularity as assessed in marrow aspirates

Case	PC $T_{½}$ min	RCU %	Non-erythroid[1]	Erythroid score[2]
N B	309	0	1	1
S Y	108	74	3	3
R W	221	2	2	2
A P	460	–	1	1
M S	247	0.3	1	1
M T	_34	6	1	2
M B	145	43	2	2
P C	98	52	1	2
N K	771	79	2	2
M J	158	62	3	2
Z P	360	12	2	2
M A	90	70	2	2
M M	134	63	3	3
A G	344	14	1	3
R M	194	52	3	3
C M	195	22	2	2

The non-erythroid component was essentially myeloid

Graded 1 3 as described in text.

The Spearman rank correlation coefficient was used for comparison between the various measures. This test was chosen in preference to parametric tests because the variances of the compared variables differed greatly With the null hypothesis that the correlation found indicated lack of correlation, one-tailed Student t test was applied to these figures.

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Table I gives the data for the analysis re lating reticulocyte count, trephine cellularity and ferrokinetic data. Tables II and III show the data relating ferrokinetic measure ments to the subjective assessment of aspirate cellularity Table IV shows the rank cor relation coefficients with their associated t and p values for the variables studied.

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Table III. Ferrokinetic data related to bone marrow aspirate myeloid and erythroid activity

		Number of cases	Mean cellularity score[2] non-erythroid[3]	erythroid
PC T	95–149 min	5	2 4	2 4
	150– 49 min	5	1 8	2 0
	250–570 min	6	1.5	1 8
RCU	0–12%	5	1 4	1.6
	13–45%	5	1.8	
	45–75%	5	2 6	6

Ferrokinetic data was graded in three arbitrarily separated groups.

Each aspirate as graded 1 3 as described in text.

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relationship was not close, and overlapping ferrokinetic results were found in groups of patients with significantly different course and prognosis. More recently *Najean and Pecking* [11] have shown that ^{59}Fe utilisation is a useful prognostic indicator for survival after the 3rd month

Because of these apparent discrepancies, the present study has been carried out to reassess the relationship of ferrokinetic measurements to the reticulocyte count and bone marrow cellularity and to the clinical severity in aplastic anaemia.

Patients and Methods

16 patients with aplastic anaemia were studied They had been referred to the Hammersmith Hospital and were thus preselected in that the referral pattern is biased to patients with severe disease. Separate analyses were made on the patients for whom complete data were available and for a group of the patients for whom the data were incomplete, either because trephine biopsy was not available, although all had marrow aspirates, or because iron utilisation was not studied for an adequate period of time.

Smears of aspirated marrow and trephine biopsies were obtained and processed by standard procedures. The cellularity of trephine biopsy sections was assessed in 11 patients by microscopy using an eyepiece with an insert which allowed view of an area 2 × 4 mm² at × 40. Four such areas were counted in each section. Because of variable cellularity in different areas of the trephine sample, an assessment was first made under low power of the overall distribution of cellular areas, and in the subsequent count the cellular areas were included in the same proportion as existed in the sample. The observer who performed the count was unaware of the results of reticulocyte counts and ferrokinetics at that time.

Bone marrow cellularity was also assessed subjectively on the smears of marrow aspirates in all cases. Attempts were made to distinguish between erythroid and non-erythroid (essentially myeloid) components. Cellularity was graded as follows.

Total cellularity

1 Fragments with only fat cells and reticulum cells

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N.B	309	0	8	256	19.5	0
M.S.	247	0.3	9	108	32.6	0.09
M.T	236	6	16	348	26.7	1.6
M.B	145	43	65	392	39.4	16.9
Z.P	360	12	13	348	17.9	2.15
A.G	344	14	10	–	21.3	2.98
N.K.	271	29	21	312	25.8	7.5
R.H.	194	52	53	–	25.8	13.4
M.J	158	62	109	124	34.0	21.1
P.C.	98	52	104	588	58.0	30.2
S.Y	108	74	60	780	68.5	50.7

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R.H.	194	52	53	–	25.8	13.4
M.J.	158	62	109	124	34.0	11
P.C.	98	52	104	588	58.0	30.2
S.Y.	108	74	60	780	68.5	50.7

Table V

Patient	Classification[1]	RCU %	Treatment	Outcome
P.C.	NS	52	androgens	alive
R.H.	NS	52	androgens	alive
A.G.	NS	14	ALG haplo-identical marrow	alive
M.B.	NS	43	BMT	dead
M.L.	NS	62	ALG haplo-identical marrow	alive
C.M.	NS	9	none	alive
L.Y.	NS	74	androgens, ALG	alive
M.A.	NS	20	none	alive
Z.P.	NS	12	BMT	dead
N.B.	S	0	BMT	alive
A.P.	S	0	ALG	dead
M.S.	S	0.3	ALG haplo-identical marrow	dead
N.K.	S	79	ALG, haplo-identical marrow	alive
M.T.	S	6	androgens	alive

Based on criteria of *Camitta* et al. [1]

NS = Not severe; S = severe; ALG = antilymphocyte globulin; BMT = bone marrow transplant. All patients received cell support where indicated.

Table VI. Retrospective analysis of prognostic criteria

	Patients alive without transplantation	Patients who died or survived with transplantation
PC $T_{1/2}$, min	204 (11)	274 (5)
RCU %	37 (10)	16 (5)
Reticulocytes, $\times 10^{10}/l$	4.8 (10)	2.4 (6)
Trephine cellularity/32 mm²	105 (4)	65 (5)

Results given are means of all patients in set. Numbers in parentheses indicate number of cases in set. The total patient number in each set varies, since all measurements were not performed in each case.

marrow cellularity from aspirated material may be misleading when there is a patchy distribution of foci of cells and that reticulocyte counts may be equally misleading especially when there is concomitant infection [4, 7] or significant dyserythropoiesis [3]. Moreover, deduction of marrow activity from reticulocyte count may be misleading in cases where there is premature release and prolonged maturation time in circulation [6]. Ferrokinetic studies provide a quantitative basis for assessing erythropoietic function and should thus provide a more reliable method for determining the extent of erythropoietic capability and indirectly the prognosis in the individual case. Accordingly, this study was carried out primarily to assess whether ferrokinetic data related to marrow cellularity and also to observe any correlation with prognosis.

When the cellularity was assessed in sections of trephine biopsy from the iliac crest or posterior iliac spine, the degree of cellularity was found to relate positively to the clearance rate of ⁵⁹Fe and to EIT but not to RCU. This discrepancy is probably due to the occurrence of ineffective erythro-

Table IV Rank correlation coefficients

	RCU	Reticulocytes	Cellularity	PIT	EIT
PC	R = 0 72 t = 3 1 n = 11 0 005<p<0 01	R = 0.81 t = 4 12 n = 11 0 0005<p<0 001	R = 0 60 t = 1 92 n = 9 0 025<p<0.05	R = 0 93 t = 7 8 n = 11 p<0 0005	R = 0.58 t = 2.15 n = 11 0.025<p<0 05
RCU		R = 0.88 t = 5.5 n = 11 p<0 0005	R = 0.56 t = 1.8 n = 9 0.05<p	R = 0.65 t = 2.58 n = 11 0.01<p<0 025	R = 0.93 t = 7 6 n = 11 p<0.0005
Reticulocytes			R = 0.396 t = 1 14 n = 9 0 05<p	R = 0 72 t = 3.06 n = 11 0.005<p<0.01	R = 0 75 t = 3 45 n = 11 0.0005<p<0.001
Cellularity (trephine)				R = 0.53 t = 1 6 n = 9 0 05<p	R = 0 61 t = 3 45 n = 9 0.005<p<0 01
PIT					R = 0 60 t = 2.26 n = 11 0 025<p<0.05

significantly to quantitation of marrow cellularity whilst PIT does not correlate with cellularity RCU is correlated with the other ferrokinetic data and with reticulocyte count, but it is not shown to correlate with marrow cellularity It should also be seen (table II III) that the qualitative assessment of total and erythroid cellularity from aspirates correlated only crudely and as a general trend with the ferrokinetic data.

In table V the clinical severity of the disease, as judged by the criteria of *Camitta* et al [1] for the purpose of patient selection for bone marrow transplantation, is correlated with RCU treatment and clinical outcome It is important to note that in some patients ferrokinetic studies were performed during the course of the disease rather than at presentation.

Table VI correlates the various parameters studied with the clinical outcomes. This shows the level of discrimination by means of which it is possible to identify those patients with less severe disease who are likely to survive without need for transplantation

Discussion

There is still much uncertainty as to what haematological tests provide the most reliable information on which to base prediction for prognosis in aplastic anaemia. It is well established that assessment of bone

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Dr. S. M. Lewis, Department of Haematology
Royal Postgraduate Medical School,
Du Cane Road, London W12 0HS (UK)

poiesis in aplastic anaemia. Subjective assessment of total non-erythroid and erythroid cellularity of aspirated bone marrow graded into three groups (1–3) also correlated in very general terms with ferrokinetic tests of overall erythroid function but less convincingly and it may be concluded that aspirates do not provide a means for reliable assessment of marrow cellularity and/or erythropoietic function in aplastic anaemia. The reticulocyte count correlated with both PC and RCU but not with trephine cellularity. *Najean* et al. [10] in a large series, were able to demonstrate that there was some correlation of cellularity with reticulocyte count and RCU but it was not close and there were considerable discrepancies.

In the present study a correlation was found between reticulocyte count and both PIT and EIT indicating that overall measurement of iron turnover reflects the peripheral manifestation of erythroid activity. The PIT (as opposed to EIT) could not be shown to correlate with marrow cellularity. This may be because its calculation is markedly influenced by the serum iron level which is affected by diurnal variables other than marrow cellularity. This does not, however explain why PIT should differ from EIT in this respect.

There are two inherent problems in attempting to correlate ferrokinetic data with prognosis. First, there may be variation in the expression of hypoplasia in the different cell lines, and secondly the severity of the disease commonly fluctuates during its course. Nevertheless, as shown in tables V and VI there was a broad correlation between RCU clinical severity and outcome of the disease. The lowest RCUs were found in those patients classified as severe by the morphological criteria used by *Camitta* et al [1]. However the importance of the variability of hypoplasia between cell lines is well demonstrated by patient MB who died of infection secondary to severe neutropenia, despite a relatively high RCU. This case illustrates that ferrokinetic data do not always provide reliable prognostic information for an individual patient. Our conclusion is that ferrokinetic studies provide useful baseline quantitative data, but alone they do not contribute significantly more than standard haematological investigations to immediate clinical management. It is possible, however that sequential ferrokinetic studies in selected patients might contribute to our understanding of the fluctuant course which is a feature of some cases.

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Dr. S. M. Lewis, Department of Haematology
Royal Postgraduate Medical School,
Du Cane Road, London W12 0HS (UK)

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determine the effects of low and high concentrations of photoactivated protoporphyrin on globin synthesis by reticulocytes, and to examine the ultrastructural alterations of the porphyrin-treated cells.

Materials and Methods

Reticulocytes

Reticulocytosis was induced by injection of phenylhydrazine into young white rabbits. Levels of up to 80% reticulocyte-enriched blood were obtained 3 days after the first injection. The reticulocytes were washed three times in phosphate-buffered saline and then suspended in Schulman's medium [12].

Determination of Globin Synthesis and Amino Acid Uptake

Packed cells, 0.02 ml, were suspended in 0.2 ml of Schulman medium containing 0.12 μCi of ^{3}H-leucine (spec. act. 400 mCi/mmol, Amersham). The reticulocytes were incubated for 15 min at 37 °C, then the cells were precipitated in hot 10% trichloroacetic acid (TCA) and the precipitate was counted [9].

For the uptake experiments 0.02 ml packed cells were suspended in 0.2 ml of medium containing 0.025 μCi of ^{3}H-α-(γ)-amino-butyric acid (GABA spec. act. 18.4 Ci/mmol, Amersham). The cells were incubated for 15 min at 37 °C, thereafter they were washed four times according to *Young* et al. [13]. The washed cells were lysed by 0.5% triton X 100 (Sigma), the proteins were precipitated by 10% TCA and sedimented. The supernatant was counted by Packard liquid scintillation counter.

Hemoglobin Determinations

Soluble hemoglobin was quantitatively measured at 540 nm, as oxyhemoglobin, by Gilford 250 spectrometer. The percentage of lysis was determined after centrifugation at 1,200 rpm for 10 min. 100% lysis was determined in samples lysed with distilled water.

Exposure of Cells to Photoactivated Protoporphyrin

Reticulocytes were incubated in Schulman's medium containing up to 60 μM of disodium protoporphyrin (Sigma). The cells were exposed to 100 watt tungsten light source, from a distance of 30 cm.

Preparation of Cells for Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

For TEM examination, reticulocytes were fixed in cold 1% glutaraldehyde in phosphate buffer pH 7.4, and postfixed in osmium tetroxide. The cells were dehydrated in graded alcohols and embedded in Epon 812. Thin sections were cut with an LKB ultratome III and examined with JEOL 100-C transmission electron microscope. For SEM examination the fixed cells were allowed to be bound to poly *L*-lysin-coated glass cover slips, then dehydrated with graded alcohols and Freons and critical point dried using CO_2. After coating with gold, the cells were examined by JEOL 35 scanning electron microscope.

Results

Figure 1 depicts the dose-dependent inhibition of photoactivated protoporphyrin on the globin-synthesizing capability of reticulocytes. 50% inhibition of globin synthesis was determined in cells exposed to 12 μM protoporphyrin and illuminated for 30 min. Under these experimental conditions, hemolysis of the cells did not exceed 10%; morphology of the cells examined by SEM and TEM showed undamaged features and the uptake of ^{3}H-GABA was reduced by only 25%. At these low concentrations, up to 15 μM of activated protoporphyrin, inhibition of protein synthesis was not a result of the lytic effect, nor of a merely reduced amino acid uptake. On the other hand, high doses of protoporphyrin, 15–60 μM induced a complete inhibition of globin synthesis, almost totally reduced amino-acid uptake and caused a 65% hemoglobin leakage.

Figure 2a shows the surface morphology

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Cross-Linking of Hemoglobin and Inhibition of Globin Synthesis in Reticulocytes Induced by Photoactivated Protoporphyrin

Z. Malik and H. Breitbart

Department of Life Sciences, Bar Ilan University, Ramat-Gan, Israel

Key Words. Globin synthesis · Hemoglobin cross-linking · Photoactivated protoporphyrin · Reticulocytes

Abstract. The photoactivated protoporphyrin effect on reticulocyte globin synthesizing capability, cross linking of intracellular hemoglobin and the cell ultrastructural alterations was studied. Low doses up to 15 μM of protoporphyrin markedly inhibited the globin synthesis and decreased amino acid uptake while no lytic effect or ultrastructural deformations were detected by scanning electron microscopy and transmission electron microscopy. On the other hand, at high doses of protoporphyrin, up to 60 μM, the globin synthesis was totally stopped, and the intracellular hemoglobin leaked out. The photodynamic effect induced cross-linking of hemoglobin into condensed spheres which captured numerous polyribosomes. The end product of protoporphyrin activity was a closed ghost with a rigid membrane containing only cross-linked hemoglobin spheres closely related to the membrane.

Introduction

Erythropoietic protoporphyria is a familial disease characterized by a rise in the red blood cell protoporphyrin concentration and hypersensitivity of the skin to sunlight. Photohemolysis of red blood cells obtained from the porphyric patients is readily induced by irradiating the cells with visible light under aerobic conditions [1]. This photodynamic process was described to be a result of the formation of singlet oxygen [2] and hydroxyl radical [3]. The activated molecules induce peroxidative degradation of polyunsaturated membrane lipids [2], aggregation of membrane particles [5], inhibition of membrane-bound enzymes [6], deterioration of active transport and cation leakage [7], cross-linking of membranal proteins and ultimately causing colloid osmotic lysis [8]. Protoporphyrin has been shown to induce an inhibition of protein synthesis and to be a potent cytotoxic agent to human granulocytes, lymphocytes and platelets [9]. Photoactivated protoporphyrin was shown to be specifically cytotoxic to various leukemic cells [10]. Furthermore, hemin and protoporphyrin in the dark had a similar effect on leukemic cells [11].

The object of the present study was to

determine the effects of low and high concentrations of photoactivated protoporphyrin on globin synthesis by reticulocytes, and to examine the ultrastructural alterations of the porphyrin-treated cells.

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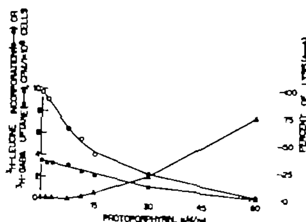


Fig. 1. The effect of photoactivated protoporphyrin on globin synthesis, ³H-GABA uptake and hemolysis of reticulocytes. Procedures in detail are described in Methods.

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TEM examination of control reticulocytes revealed the typical components of a reticulocyte. The cells were hemoglobinized, and contained many polyribosomes and residual mitochondria (fig. 3a). By exposure of these cells to the photoactivated protoporphyrin dark masses of condensed hemoglobin appeared in the cytoplasm (fig. 3b).

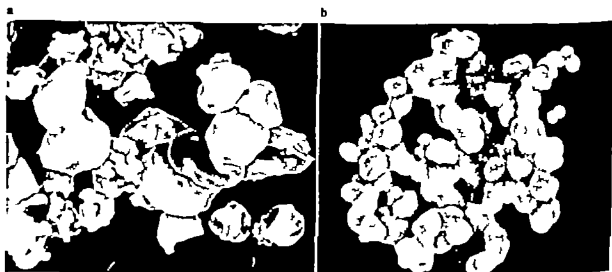
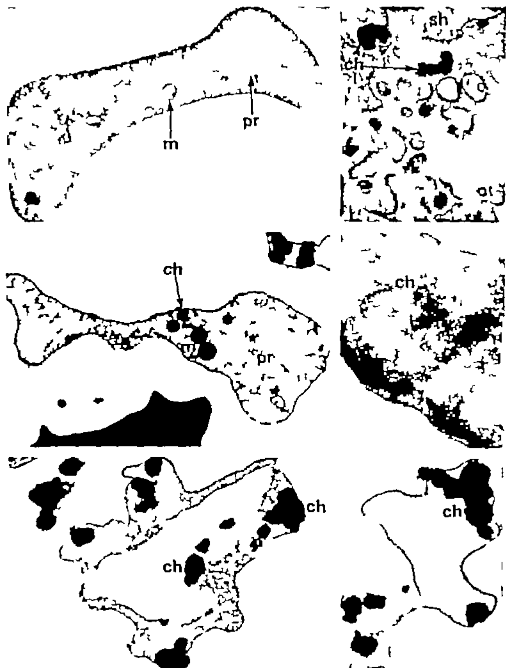


Fig. 2. Induction of reticulocyte deformation by photoactivated protoporphyrin SEM examination. a Control reticulocyte. × 2,300. b Deformation of a reticulocyte by the photodynamic effect. × 13,200.

Fig. 3. Ultrastructural features of intracellular events induced by photoactivated protoporphyrin. TEM examination. a Control reticulocyte: polyribosome (pr) and mitochondria (m) are indicated. × 23,800. b Protoporphyrin-treated cells, condensed masses of hemoglobin (ch) and soluble hemoglobin (sh), which leaked outside the cells, are indicated. × 10,000. c The affected reticulocyte retained mitochondria (m), polyribosomes (pr) and residual amounts of soluble hemoglobin. × 9,300. d The condensed hemoglobin spheres contain captured polyribosomes. × 65,000. e At the cell projections hemoglobin spheres exist and contribute to the bleb shape seen by SEM. × 13,600. f A typical ghost – the end-product of the photodynamic effect – containing hemoglobin spheres surrounded by a rigid membrane. × 13,500.


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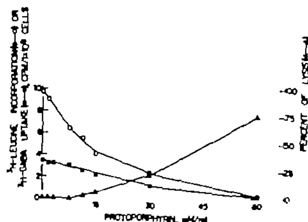


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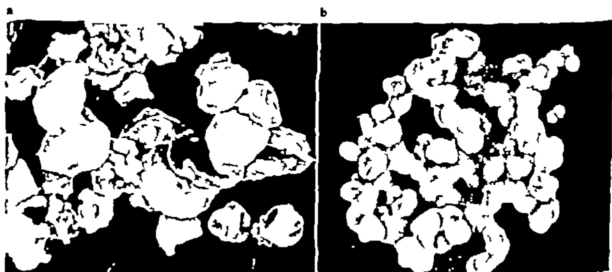
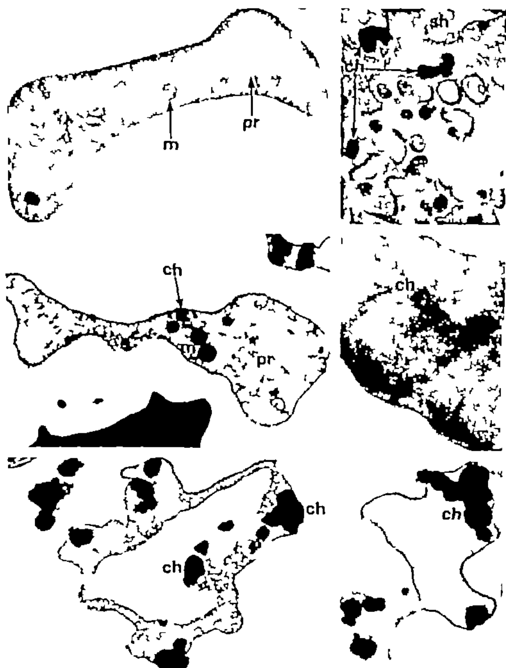


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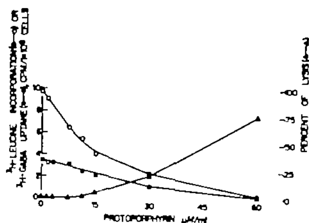


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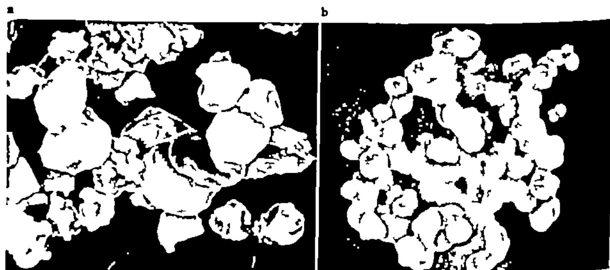


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tocytoma cells, and myelocytic leukemia cells [10] These cell lines were affected intracellularly or at the surface by the photoactivated protoporphyrin.

The various effects of propbyrins on normal and leukemic cells is of theoretical and practical importance. The present study demonstrates specific effects of protoporphyrin, which can be applied for more complicated processes.

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Dr Z. Malik, Department of Life Sciences, Bar-Ilan University Ramat-Gan (Israel)

Thereafter most of the soluble hemoglobin leaked outside the cells, while polyribosomes and mitochondria were intracellularly retained (fig. 3c) It is conceivable that the condensed hemoglobin spheres are a product of the cross-linking of hemoglobin induced by the photodynamic effect. Occasionally it was detected that the hemoglobin spheres contained a large number of polyribosomes captured through the hemoglobin cross-linking process (fig. 3d) The intermediate product was a deformed cell containing only residual amounts of soluble hemoglobin while condensed hemoglobin particles were randomly distributed on the cell projections (fig. 3e) The end-product of the photodynamic effect was a closed ghost with a rigid membrane, containing only the cross linked hemoglobin spheres bound to the membrane (fig. 3f) The end-proudct was free of ribosomes, whereas nitochondria were detected in numerous cells.

Discussion

The present study demonstrates distinct biochemical and morphological events in reticulocytes exposed to photoactivated protoporphyrin The results show an inhibitory effect on globin synthesis induced by 5–15 μM of protoporphyrin, which was shown to be independent of the lytic effect. The reduced rate of GABA uptake may contribute to the magnitude of the inhibition although it was probably not the main reason for it. The inhibition of globin synthesis at such low concentrations of protoporphyrin may result from a direct interference effect to the recycling of the initiation proteins, controlled by hemin [14] Other possible explanations for the inhibitory effect are the damaged membrane releases an inhibitor which interferes with the translation mechanism [15] reduced levels of potassium in the cells [7] or direct conformational damage to the polyribosomes.

Hemoglobin leakage which characterizes the lytic phenomena, was shown to be stimulated by the high levels of protoporphyrin 15–60 μM The leaky membrane is possibly a direct result of cross-linking of membranal proteins [7 8] The possibly existing holes in the membrane retained the ribosomes and mitochondria, while hemoglobin diffused outside the cell

The observation of the appearance of condensed hemoglobin spheres, which possibly stems from intracellular hemoglobin cross-linking, is a new ultrastructural finding. It was described that membranal proteins were cross linked by the photoactivated protoporphyrin, while no other intracellular proteins were assumed to be corss-linked [5–8] The particular hemoglobin globules were closely related to the membrane, which may explain the sponge-like shape seen by the SEM The blebs at the end of the cytoplasmic projections are shaped by the hemoglobin-condensed globules.

The photodynamic effect of porphyrins was successfully applied for cancer therapy in humans [16] Hematoporphyrin was shown to accumulate in various tumors and exposure of the tumor area to visible light induced a selective destruction of the malignant tissue [16] No side effects were reported. Recently we have shown a selective destruction of chronic lymphocytic leukemia lymphocytes by hemin and protoporphyrin in the dark [11] In addition, we have shown distinct sites for protoporphyrin accumulation by Friend erythroleukemia cells, Burkitt lymphoma lymphocytes, mas-

estigate whether this molecule, similarly to other sulphydryl compounds, is also able to modify the RBC membrane, inducing PNH features.

Materials and Methods

Normal ... *blood* anticoagulated with heparin, was collected from healthy volunteers, the RBC (O Rh+) were washed three times in 0.9% cold NaCl to remove the plasma and the buffy coat, and used for the experiments immediately (For each experiment RBCs from different donors ere used.)

PNH RBC were obtained from PNH patient, in which the diagnosis was confirmed by the positivity of Ham and sugar tests and the reduction of AChE activity

PNH-lik RBCs were prepared by incubating normal RBCs with AET 0.143 *M* pH 8.5, for 30 min at 37 C following the method described by *Sirchia* et al. [5] These cells displayed positive Ham and sugar tests and reduction in AChE activity

The acidified serum (Ham) test and *the sugar test* ere performed as suggested by *Dacie and Lewis* [2] *AChE activity* of RBCs was measured using the spectrophotometric method of *Weber* [6] *Radioactive choline* (^{3}HCh, 2 Ci/mmol) and *acetylcholine* (A-^{3}HCh, 200 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, Great Britain), and diluted t final concentration of 25 × 10^{-3}*M* *2 MPG* was supplied by Sigma.

Procedure 1 vol of packed RBCs was incubated at 37 C with 4 vol of different solutions of 2 MPG, for variable times (15 30, 45 and 60 min) The 2 MPG was used at the concentrations of 0.150, 0.300 and 0.450 *M* and at the pH of 7.5 8, 8.5 and 9 After the incubation time, the RBCs ere washed three to four times with cold saline to remove the substance and, if present, haemolysis, and were used immediately for the subsequent tests The uptake of radioactivity in the presence of labelled choline or acetylcholine was performed as previously described [4]. The experiments were done in triplicate, and the results expressed as mean of values.

Results

Normal RBCs after incubation with 2-MPG solution, pH 8.5 for 30 min, displayed a positivity of Ham and sugar tests. The positivity of both tests increased directly with the concentration of the substances (fig. 1) and the increase of pH (fig. 2) The lower lysis in Ham and sugar tests of the

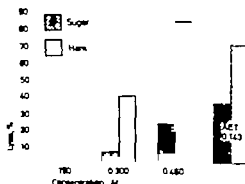


Fig. 1. Haemolysis in Ham's test and the sugar test after 30 minutes incubation of normal RBC with 2 MPG pH 8.5, at different concentrations The effect of AET 0.143 *M* is reported in comparison.

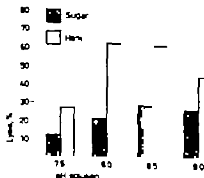


Fig. 2. Haemolysis in Ham test and the sugar test after 30 minutes incubation of normal RBC with -MPG 0.450 *M* at different pH.

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In vitro Production of PNH-Like Red Blood Cells by 2-Mercaptopropionylglycine[1]

G C Falezza R Corrocher O Olivieri F Capra E Trevisol A Pennacchioni and G De Sandre

Istituto di Patologia Medica II (Dir., Prof G *De Sandre*) Universita di Padova, Verona, Italy

Key Words. Choline transport Erythrocyte acetylcholinesterase 2 Mercaptopropionylglycine Paroxysmal nocturnal haemoglobinuria

Abstract. 2 Mercaptopropionylglycine (2 MPG) transformed normal red blood cells (RBCs) into paroxysmal nocturnal haemoglobinuria (PNH)-like RBCs in vitro depending on the concentration pH and time of incubation. The incorporation of radioactive choline in the presence of acetylcholine was reduced, as in RBCs treated with aminoethylisothiouronium salt (AET) In contrast, the uptake in the presence of choline differed when the RBCs were incubated with the two compounds, being reduced in AET treated RBCs and increased in 2 MPG-treated ones. As true PNH RBCs incorporated to a higher extent the radioactivity in the presence of both acetylcholine and choline 2 MPG-treated RBCs seemed to resemble the PNH RBCs better than the AET treated ones Present results suggest the possibility of modifying selectively the activity of acetylcholinesterase and the transport of choline through the cell membrane

Many sulphydryl compounds have been demonstrated to transform in vitro normal human red blood cells (RBCs) into paroxysmal nocturnal haemoglobinuria(PNH) like RBCs [3 5] The PNH-like RBCs share with true PNH RBCs the in vitro sensibility to complement lysis, as exhibited by the positivity of Ham (acid lysis) and sugar tests, and the reduction of the acetylcholinesterase (AChE) activity

We have recently demonstrated that, in contrast to true PNH RBCs, the PNH-like RBCs obtained by treatment with aminoethylisothiouronium salt (AET) displayed an impaired in vitro ability to take up choline from the incubation medium in the presence of both choline or acetylcholine [1 4]

2 Mercaptopropionylglycine (2-MPG) is a sulphydryl compound mainly used in the treatment of some hepatic diseases as a sulphydryl group donor It has also been reported to be of some beneficial effect in pyruvate kinase (PK) deficiency anaemia, possibly through a modification of the redox state of the thiol group in red blood cells [7] The aim of the present study is to in-

[1] Supported by CNR grant 78 02078 04

Both 2-MPG- and AET-treated RBCs showed a different behaviour than PNH RBCs, in which the uptake of the radioactivity in the presence of acetylcholine was greatly enhanced (fig. 5).

In contrast, when the incubation was carried out in the presence of labelled choline, the uptake of radioactive substance by 2 MPG RBCs was greatly exalted, as in PNH RBCs, whereas the uptake by AET RBCs remained depressed (fig. 6).

Discussion

Our results show that 2 MPG as many other sulphydryl compounds, is able to transform in vitro normal RBCs into PNH-like RBCs, depending on the concentration, pH and time of incubation. (Differences in / lysis under identical conditions between figures 1 and 2 or 3 respectively can be attributed to the use of RBCs from different donors.)

PNH-like RBCs share with true PNH RBCs the reduction of AChE activity and the susceptibility to complement lysis as demonstrated by the positivity of Ham and sugar tests. Nothing is known about the biochemical modification of the membrane neither in PNH RBCs nor in PNH like RBCs, however the similarity between the two kinds of cells is only based on the presence of the above-mentioned laboratoristical markers.

In a previous report [4] we have stressed that the uptake of radioactivity by RBCs in the presence of acetylcholine implied the cleavage of the molecule by the enzyme AChE, the membrane being impermeable to acetylcholine but not to choline. Moreover we have recently demonstrated [1] that, in

Table I. Differences between PNH and PNH-lik RBC

Type of RBCs	AChE	Uptake the presence of acetylcholine	choline
PNH	↓	↑	↑
-MPG-treated	↓	↓	↑
AET-treated	↓	↓	↓

contrast to AET-treated RBCs, which showed an impaired ability to take up choline in the presence of both choline and acetylcholine, true PNH RBCs showed an opposite behaviour namely an increased ability to take up radioactivity in the presence of both substances.

The experiments performed with 2-MPG show an intermediate behaviour the uptake of radioactivity being reduced in the presence of acetylcholine and increased in the presence of choline. This implicates that different types of PNH-like RBCs can be produced in vitro using different sulphydryl compounds and that no one of them is really the same as PNH RBCs (table I).

Moreover it results clearly that the low level of AChE activity is not a limiting factor in the uptake of choline in the presence of acetylcholine, the residual activity being largely sufficient for the splitting of the molecule.

Since the increased uptake of choline was also noted in reticulocytes [1], it was suggested that the raised incorporation of choline in PNH RBCs was attributable to the presence of a younger red cell population in this situation. The findings obtained here suggest that the uptake of choline may be increased by a structural modification of the RBC membrane independent of the age

sample incubated at pH 9 was probably attributable to the remarkable spontaneous lysis observed during incubation with 2 MPG suggesting that many sensitive cells were destroyed.

The effect on RBCs of the time of incubation with 2 MPG 0.3 *M* is expressed in figure 3. The prolongation of incubation caused an increase in the positivity of both Ham and sugar tests.

The AChE activity was partially inhibited by the treatment with 2 MPG (0.450 *M* pH 8.5 for 30 min) as shown in figure 4.



Fig. 3. Haemolysis in Ham's test and the sugar test after incubation for different times of normal RBCs with 2 MPG 0.300 *M* pH 8.5.

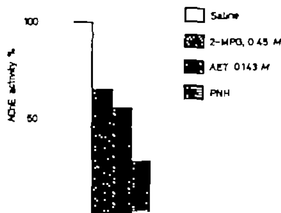


Fig. 4. AChE activity in normal RBCs (incubated with saline) and in RBCs with either 2 MPG (0.450 *M* pH 8.5) or *AET (0.143 M pH 8.5)*.

When incubated with labelled acetylcholine ($6 \cdot 10^{-4}$ *M*) 2 MPG-treated (0.300 *M* for 30 min) RBCs displayed an impaired ability to take up the radioactivity but to a lesser extent than AET-treated (0.143 for 30 min) RBCs.

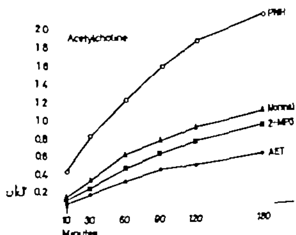


Fig. 5. Uptake of choline, in the presence of labelled acetylcholine, by different types of RBCs (normal, PNH-treated, 2 MPG-treated and AET treated). C_i/C_e refers to the ratio between the concentration of radioactivity inside and outside the cell.

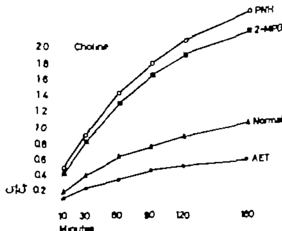


Fig. 6. Uptake of choline, in the presence of *labelled choline, by different types of RBCs (legend as in fig. 5)*.

Both 2-MPG- and AET-treated RBCs showed a different behaviour than PNH RBCs, in which the uptake of the radioac tivity in the presence of acetylcholine was greatly enhanced (fig. 5).

In contrast, when the incubation was carried out in the presence of labelled choline, the uptake of radioactive substance by 2 MPG RBCs was greatly exalted, as in PNH RBCs, whereas the uptake by AET RBCs remained depressed (fig. 6).

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Type of RBCs	AChE	Uptake in the presence of	
		acetylcholine	choline
PNH	↓	↑	↑
MPG-treated	↓	↓	↑
AET treated	↓	↓	↓

Discussion

Our results show that 2 MPG as many other sulphydryl compounds, is able to transform in vitro normal RBCs into PNH-like RBCs, depending on the concentration, pH and time of incubation. (Differ ences in / lysis under identical conditions between figures 1 and 2 or 3 respectively can be attributed to the use of RBCs from different donors.)

PNH like RBCs share with true PNH RBCs the reduction of AChE activity and the susceptibility to complement lysis as demonstrated by the positivity of Ham and sugar tests. Nothing is known about the biochemical modification of the membrane neither in PNH RBCs nor in PNH-like RBCs however the similarity between the two kinds of cells is only based on the presence of the above-mentioned laboratoristical markers.

In a previous report [4] we have stressed that the uptake of radioactivity by RBCs in the presence of acetylcholine implied the cleavage of the molecule by the enzyme AChE, the membrane being impermeable to acetylcholine but not to choline. Moreover we have recently demonstrated [1] that, in contrast to AET-treated RBCs, which showed an impaired ability to take up choline in the presence of both choline and acetylcholine, true PNH RBCs showed an opposite behaviour namely an increased ability to take up radioactivity in the presence of both substances.

The experiments performed with 2-MPG show an intermediate behaviour the uptake of radioactivity being reduced in the presence of acetylcholine and increased in the presence of choline. This implicates that dif ferent types of PNH-like RBCs can be produced in vitro using different sulphydryl compounds and that no one of them is real ly the same as PNH RBCs (table I).

Moreover it results clearly that the low level of AChE activity is not a limiting fac tor in the uptake of choline in the presence of acetylcholine, the residual activity being largely sufficient for the splitting of the molecule

Since the increased uptake of choline was also noted in reticulocytes [1] it was suggested that the raised incorporation of choline in PNH RBCs was attributable to the presence of a younger red cell population in this situation. The findings obtained here suggest that the uptake of choline may be increased by a structural modification of the RBC membrane independent of the age

of the cells, so that we cannot exclude a similar selective modification in PNH RBCs.

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Dr Giancarlo Falezza,
Istituto di Patologia Medica II
Policlinico di Borgo Roma,
37100 Verona (Italy)

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Investigation of the Correlation between Total Blood Potassium Concentration and Hematocrit

G Buzzigoli, M Gonella W Bencivelli V Bartolini and G Betti

Laboratorio di Fisiologia Clinica, CNR, Divisione Nefrologica, Spedali Riuniti, Pisa, and Sezione Emodialisi, Ospedale di Volterra, Volterra Italy

Key Words. Erythrocyte potassium concentration Hematocrit Whole blood potassium concentration

Abstract. The correlation between whole blood potassium concentration (Kt) and hematocrit (Ht) was investigated in subjects with a broad range of Ht. This relationship was found to be represented by a curve, and its form was derived by the manipulation of the formula concerning the inverse correlation between Ht and erythrocyte potassium concentration (Kc)

Introduction

Potassium is the most representative intracellular cation, and it is well established that its whole blood concentration (Kt) is related to the red cell count [1] In our previous works [2, 3] an inverse linear relationship between erythrocyte potassium concentration (Kc) and hematocrit (Ht) was found, when the data of both healthy and anemic patients were considered simultaneously This linear correlation is independent of plasma potassium level (Kp) hemoglobin concentration and reticulocyte count. This relationship is represented by equation 1 [2]

$$Kc = a - bHt, \qquad (1)$$

where $a = 116.64 \pm 2.70$ and $b = 59\ 71 \pm 7\ 16$.

In view of the standard equation

$$Kt = KcHt + Kp\ (1-Ht),$$

Kc can also be expressed [1] as.

$$Kc = \frac{Kt - Kp\ (1-Ht)}{Ht} \qquad (2)$$

By combining equations 1 and 2 as follows:

$$a - bHt = \frac{Kt - Kp\ (1-Ht)}{Ht}$$

it is possible to derive, by algebraic manipulation, an equation to express Kt in terms of Ht and Kp·

$$Kt = Kp + (a-Kp)Ht - bHt^2 \qquad (3)$$

Because the clinical range of values of Kp is small compared to Kt, the variations

of the cells so that we cannot exclude a similar selective modification in PNH RBCs.

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Table I. Measured values of Ht, Kt and Kp in group 1

No.	Ht expressed as fraction of 1	Kt mEq/l	Kp mEq/l
1	0.17	19	3.3
2	0.21	25	4.0
3	0.22	25	4.0
4	0.23	28	3.5
5	0 4	27	4.1
6	0.25	29	3.7
7	0.27	30	4.2
8	0.27	33	4.7
9	0.28	30	3.0
10	0.31	32	2.9
11	0.31	34	4.0
12	0.31	32	4.3
13	0.32	33	3.5
14	0.33	35	3.85
15	0.33	35	3.8
16	0.34	38	4.4
17	0.34	36	3.65
18	0.35	36	3.45
19	0.35	37	3.65
20	0.35	35	3.75
21	0.35	38	3.7
22	0.36	33	2.5
23	0.36	35	3.75
4	0.37	35	4.15
25	0.37	36	3.65
26	0.38	39	3.8
27	0.38	36	4.0
28	0.40	40	3.3
29	0.40	40	3.6
30	0.40	41	3.2
31	0.42	40	3.75
32	0.43	40	3.95
33	0.43	43	4.3
34	0.45	42	4.1
35	0.45	43	3.95
36	0.46	44	3.7
37	0.47	42	4.5
38	0.47	42	4.2
39	0.47	42	4.0
40	0.48	44	4.45
41	0.50	44	4.15
42	0.51	46	4.6
43	0.52	49	4.0
44	0.54	48	4.8

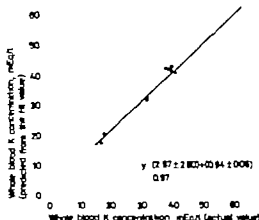


Fig. 2. Correlation between predicted and measured Kt values in group 2.

Analysis of the Predictive power (Group 2)

In group 2 we found a close correlation (r = 0.97) between the 15 actual values of Kt and those predicted from equation 5 This correlation is shown in figure 2. The intercept of the regression line is not significantly different from zero and the slope from 1 indicating that the predicted value can be substituted for the measured value. The measured values in group 2 for Ht, Kp and Kt, and the values of Kt derived from equation 5 are shown in table II.

Consistency of the Regression Parameters

Although the parameters α, β and γ have been derived directly from data, it is to be expected, by comparing equations 3 and 4 that the values of these parameters lie respectively close to $\bar{K}p$, (a–$\bar{K}p$) and b where the values for a and b in equation 1 were computed independently in equation 2. As shown in table III the value of α lies within 1 SD of $\bar{K}p$, and furthermore, β and

in Kp do not significantly affect the magnitude of Kt calculated using equation 3 therefore we would expect that ignoring the dependence of equation 3 on Kp would not alter its predictive power This means that for the purpose of Kt evaluation Kp can be assumed to be an unknown constant value, without adding a significant error

As a matter of fact a deviation of the actual value of Kp from a constant value will affect in opposite ways the first two terms of equation 3 (i.e one will be increased while the other will be reduced) thus providing a partial compensation in the propagation of errors On this basis, Kt can be expressed as a quadratic function of Ht alone for this purpose we assume that

$$Kt = \alpha + \beta Ht - \gamma Ht \quad (4)$$

In this study α β and γ have been estimated directly by least square quadratic regression from the measured values of Kt and Ht, and not derived from a and b (equation 1) or from the measured values of Kp

Patients and Methods

44 subjects (group 1) with a broad range of Ht were examined. The normal subjects were members of the hospital staff The anemic patients were suffering from chronic debilitating diseases, their anemia being normochromic. The Ht was obtained in duplicate by the standard macromethod of *Wintrobe* [4] Kt and Kp were determined by atomic absorption spectrophotometry [5]. The actual coefficients of variation of these parameters were 0.5% for Ht, 1.5% for Kp and 2.0% for Kt. The constants α, β and γ in formula D were derived using the values of Kt and Ht in these patients (group 1). The values of Kp were used only to test the consistency of α, β and γ in terms of Kp (a–Kp) and b as in *equation* 3 In addition we tested the predictive power of equation 4 a second group of 15 subjects (group 2) was selected with a similar range of Ht. In these subjects the Kt was measured and each value was compared with the corresponding value obtained from equation 4 using the individual Ht and the values of α, β and γ derived from group 1

Results

Analysis of the Correlation between Kt and Ht (Group 1)

The correlation between Kt and Ht in group 1 was found to be represented by the curve depicted in figure 1 This correlation is expressed by equation 5

$$Kt = 3.73 + 112.63\ Ht - 59.27\ Ht^2 \quad (5)$$

computed by second-order polynomial approximation using the least square method, analysis of variance has shown an F value of 378 38 with 41 vs. 2 d.f The measured values of Ht, Kt and Kp are reported in table I

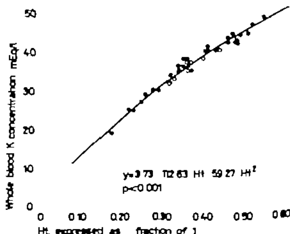


Fig. 1. Relationship between Kt and Ht in group 1.

Table I. Measured values of Ht, Kt and Kp in group 1

No	Ht expressed as fraction of 1	Kt mEq/l	Kp mEq/l
1	0.17	19	3.3
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8	0.27	33	4.7
9	0.28	30	3.0
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13	0.32	33	3.5
14	0.33	35	3.85
15	0.33	35	3.8
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18	0.35	36	3.45
19	0.35	37	3.65
20	0.35	35	3.75
21	0.35	38	3.7
22	0.36	33	2.5
23	0.36	35	3.75
24	0.37	35	4.15
25	0.37	36	3.65
26	0.38	39	3.8
27	0.38	36	4.0
28	0.40	40	3.3
29	0.40	40	3.6
30	0.40	41	3.2
31	0.42	40	3.75
32	0.43	40	3.95
33	0.43	43	4.3
34	0.45	42	4.1
35	0.45	43	3.95
36	0.46	44	3.7
37	0.47	42	4.5
38	0.47	42	4.2
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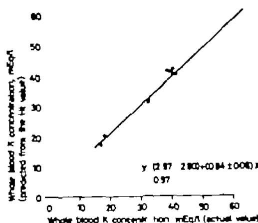


Fig. 2. Correlation between predicted and measured Kt values in group 2

Analysis of the Predictive power (Group 2)

In group 2 we found a close correlation ($r = 0.97$) between the 15 actual values of Kt and those predicted from equation 5. This correlation is shown in figure 2. The intercept of the regression line is not significantly different from zero and the slope from 1, indicating that the predicted value can be substituted for the measured value. The measured values in group 2 for Ht, Kp and Kt, and the values of Kt derived from equation 5 are shown in table II.

Consistency of the Regression Parameters

Although the parameters α, β and γ have been derived directly from data, it is to be expected, by comparing equations 3 and 4, that the values of these parameters lie respectively close to $\bar{K}p$ $(a-\bar{K}p)$ and b, where the values for a and b in equation 1 were computed independently in equation 2. As shown in table III the value of α lies within 1 SD of $\bar{K}p$, and furthermore, β and

Table II. Measured values of Ht, Kp and Kt, and predicted values of Kt in group 2

No.	Ht expressed as fraction of 1	Kp mEq/l	Kt actual value mEq/l	Kt predicted value mEq/l
1	0 13	3 4	17	17.37
2	0.16	3 45	18	20_3
3	0 17	4 8	23	21 16
4	0.29	4.2	32	31 41
5	0.30	3.8	32	32.18
6	0.31	3.5	35	32.95
7	0 42	4 75	40	40.58
8	0.42	3 8	41	40.58
9	0 42	3 8	43	40.58
10	0 43	3.8	39	41.20
11	0 44	4.3	38	41.81
12	0 45	3 65	40	42.41
13	0 48	4 75	41	44 16
14	0.55	4 1	44	47 75
15	0 69	4 4	59	53.23

Table III. Comparison between coefficients derived from a, b and $\bar{K}p$ and estimated by least square analysis

Coefficient derived from a, b Kp	Coefficient by least square analysis
$\bar{K}p$ = 3 85 ± 0.46	α = 3 73
a $\bar{K}p$ = 112.79 ± 2.74	β = 112.63
b = 59 7 ± 7 16	γ = 59.27

γ lie within 1 SD of (a–$\bar{K}$p) and b, respectively

Discussion

It has previously been shown [2, 3] that an inverse linear relationship between Kc and Ht exists, a decrease in Ht being partially compensated by elevation of the erythrocyte K concentration. This suggests that the relationship between Kt and Ht is not linear In this study it has been shown, by exploring a wide range of Ht values, that this relationship can be expressed as a second-order polynomial curve whose coefficients can be estimated. It can be concluded that, using this equation, the Kt can be accurately calculated directly from the patient s Ht value.

Acknowledgement

The authors are deeply indebted to Dr *G J Davies* for helpful discussion in revising the manuscript.

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G Buzzigoli, Head, Clinical Chemistry Unit, Clinical Physiology Laboratory Via Savi, 8, I 56100 Pisa (Italy)

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Chronic Myeloproliferative Disorders
A Quantitative Assessment of Platelet Ultrastructure

B J Boughton D W Jerrome and M Rychetnik

Departments of Haematology and Pathology John Radcliff Hospital, Headington, Oxford, England

Key Words. Aspirin Morphology Myeloproliferative Platelet storage pool

Abstract. In patients with chronic myeloproliferative disorders (MPD) and impaired in vitro platelet aggregation, a quantitative assessment of platelet ultrastructure showed reduced numbers of α granules and excessive dilation of the open canalicular systems. After 10 days of aspirin therapy some abnormalities were significantly reduced. The implications of this finding are discussed.

Introduction

Morphological abnormalities of the myeloproliferative blood platelet have been known for more than half a century [1] Since then, electron microscopy has revealed a wide range of ultrastructural defects. Minor reported abnormalities include hypertrophy of the dense tubular systems, persistent rough endoplasmic reticulum and prominent glycogen granules. These features are restricted to a small percentage of cells, however and since they are also seen occasionally in normal platelets, their significance is doubtful [2] A number of studies have reported additional, more widespread structural defects, the most consistent of which are a decrease in the number of granules and excessive dilation of the open canalicular systems (OCS) [3–5] The first reflects the platelet storage pool deficit of ADP and 5HT [6] but the nature of the dilated OCS has remained obscure. Since the OCS is the pathway for the release of granules, and since it is dilated in experimentally activated platelets [7] this morphological appearance supports the recent proposal that MPD platelets have acquired a storage pool defect due to intravascular platelet activation [8] This challenges the proposal that MPD platelet abnormalities are directly attributable to dysplastic, malignant megakaryocytopoiesis [9 10]

In the following study we have developed an objective method for the assessment of these morphological platelet abnormalities, and have used this to measure the effect of aspirin therapy on platelet morphology Aspirin has been shown not to alter platelet morphology via a direct effect

Table II. Measured values of Ht, Kp and Kt, and predicted values of Kt in group 2

No.	Ht expressed as fraction of 1	Kp mEq/l	Kt actual value mEq/l	Kt predicted value mEq/l
1	0 13	3 4	17	17.37
2	0.16	3 45	18	20.–3
3	0 17	4 8	–3	21 16
4	0.29	4.2	32	31 41
5	0.30	3 8	32	32.18
6	0.31	3.5	35	32.95
7	0.42	4 75	40	40.58
8	0 42	3.8	41	40.58
9	0 42	3.8	43	40.58
10	0.43	3.8	39	41.20
11	0 44	4.3	38	41.81
12	0.45	3 65	40	4–.41
13	0.48	4 75	41	44 16
14	0.55	4 1	44	47 75
15	0 69	4 4	59	53.23

Table III. Comparison between coefficients derived from a b and $\overline{K}p$ and estimated by least square analysis

Coefficient derived from a, b, Kp	Coefficient by least square analysis
$\overline{K}p$ = 3.85 ± 0 46	α = 3 73
a–$\overline{K}p$ = 112.79 ± 2.74	β = 112.63
b = 59 7 ± 7 16	γ = 59 27

γ lie within 1 SD of (a–$\overline{K}p$) and b respec tively

Discussion

It has previously been shown [2, 3] that an inverse linear relationship between Kc and Ht exists, a decrease in Ht being par tially compensated by elevation of the eryth-rocyte K concentration. This suggests that the relationship between Kt and Ht is not linear In this study it has been shown, by exploring a wide range of Ht values, that this relationship can be expressed as a sec ond-order polynomial curve whose coeffi-cients can be estimated It can be concluded that, using this equation, the Kt can be ac curately calculated directly from the pa-tient s Ht value.

Acknowledgement

The authors are deeply indebted to Dr *G J. Davies* for helpful discussion in revising the manu-script.

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history of splenic infarction, superficial venous thrombosis or iliac artery thrombosis. None were on anticoagulant therapy but all 5 cases showed a marked reduction in 1 and 2 wave adrenaline-induced platelet aggregation.

Platelet α Granules

Figure 2 shows that platelet granulation varied from 0 to 40% of the cross-sectional area, both in patients and normal controls. The most frequent (modal) value for normal platelets was 15–20%. In contrast, the patients platelets show an excess of poorly granulated cells with a modal value of 0–5%. When assessed by chi-squared, the patients and normal platelets showed highly significant differences in distribution ($p < 0.001$).

Platelet OCS

Figure 3 shows variable OCS dilation in both patients and normal controls. The modal value for controls is 10–15% and for the patients 15–20%, and the entire distributions are also statistically different ($p < 0.001$)

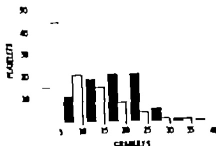


Fig. 2. The % of platelets with granules occupying 0–45% of their total area. The shaded and open colums show the mean values for 3 normal subjects and 5 patients, respectively. The distribution of granules is significantly reduced in the patients ($p < 0.001$).

Aspirin Effect

Figure 4 shows that after aspirin, there was a decrease in poorly granulated cells. However the appearances have not returned to the normal spectrum shown in figure 2, and the modal granulation value of 0–5% was the same before and after aspirin. Despite this, granulation is significantly improved overall ($p < 0.001$). Figure 5 shows that in addition, the excess fo cells with

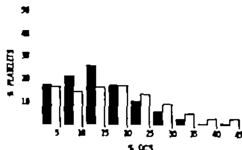


Fig. 3. The % of platelets with OCS occupying 0–45% of their total area. Normal subjects and patients are displayed as in figure 2. The distribution of OCS is statistically increased in the patients ($p < 0.001$).

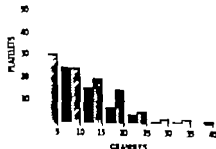


Fig. 4. The % platelets with granules occupying 0–40% of their area. The shaded and hatched columns show mean values for 3 patients before and after aspirin, respectively. Granulation is increased after aspirin therapy ($p < 0.001$), but has not returned to normal.

on the megakaryocytes [11] whereas it does prevent in vivo MPD platelet storage pool release [17]

Methods

Patients and Controls

The diagnosis of myeloproliferative disease has been previously discussed [12] 4 of the 5 patients were on myelosuppressive chemotherapy but none were permitted aspirin or similar drugs for 10 days prior to their initial assessment. Following this, they were given 1,200 mg aspirin daily for 10 days and then reassessed. The normal controls were healthy laboratory personnel.

Electron Microscopy (E/M)

Citrated platelet-rich plasma was fixed with 0 1% glutaraldehyde prior to the production of platelet buttons by centrifugation at 1,500 g This technique produces minimal alteration in platelet morphology [13] The platelet buttons were then fixed further with 3% glutaraldehyde. They were then macroscopically sectioned into 6 random blocks, which were then randomly mounted for microscopic sectioning and processed for E/M as described elsewhere [13] The E/M grids were scanned randomly in battlement fashion and photographed at × 33 000 final magnification in a Phillips E/M

Ultrastructural Assessment

The photographs were mounted on transparent grids (fig. 1), and the cross-sectional area of each ultrastructural component was computed from the number of points overlying each structure [14]. About 100 platelets were analysed in each subject (range 99–113 cells) Since the mean total cross-sectional area of each cell was about 72 grid points (range 10–437), between 6,000 and 7,000 total grid points were counted in each patient. Table I shows that the variation inherent to the method depends on the cell size and the proportion of the cell occupied by the structure under consideration. These variations produce distributions for individual patients which do not vary significantly (chi-squared test $p > 0.5$)

Platelet Aggregation

These methods have been previously described [12].

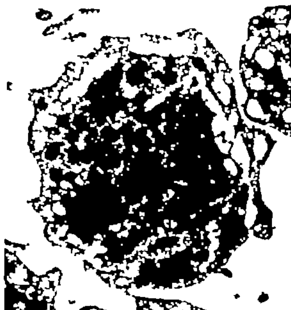


Fig. 1 E/M of a platelet overlaid with the transparent counting grid × 33,000.

Table I. Granules and OCS in platelets were each measured 10 times the variation in the counting procedure is small and depends on the cell size, and the size of the ultrastructural component

Cell size		Mean ± 1 SD
Procedure 1		
63	granules	2.89 ± 1 6
63	OCS	32.82 ± 3.9
Procedure 2		
120	granules	3 42 ± 0 8
120	OCS	29 0 ± [illegible]5

Statistics

Differences between the distributions of granules and OCS were assessed using the chi-squared test.

Results

Patients

None had current thrombotic or haemorrhagic complications but 3 cases had a past

history of splenic infarction, superficial venous thrombosis or iliac artery thrombosis. None were on anticoagulant therapy but all 5 cases showed a marked reduction in 1 and 2 wave adrenaline-induced platelet aggregation.

Platelet α Granules

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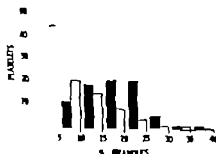


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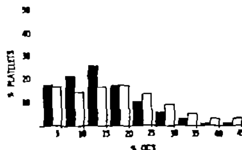


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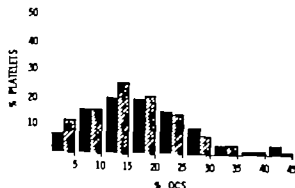


Fig. 5 The % platelets with OCS occupying 0–45% of their area. The shaded and hatched columns indicate mean values for 3 patients before and after aspirin therapy respectively. No significant change is present ($p > 0.5$) but cells with very prominent (40–45%) OCS reduced after aspirin.

prominent (40–45%) OCS was eliminated by aspirin, though the overall distribution of OCS is statistically unchanged ($p < 0.5 > 0.1$).

Discussion

The subjective analysis of platelet ultrastructure is difficult, due to the variation in the appearance of normal as well as abnormal cells. The quantitative method used in this present study of MPD confirms, however, the previous subjective impressions of α granule depletion and OCS dilation.

Definite improvements in morphology occurred after aspirin therapy but the changes were not impressive enough to resolve the conflicting views on the pathogenesis of the MPD platelet defect. Aspirin would be expected to protect MPD platelets from intravascular storage pool release since the drug relieves symptoms of platelet embolization, reduces circulating platelet aggregates and inhibits the intravascular release of platelet β-thromboglobulin [15–17]. Aspirin does not reduce the hypersensitivity of MPD platelets caused by the failure of PgD_2 inhibition [18, 19]. Furthermore, it does not prolong platelet survival in states of increased platelet consumption, and its antithrombotic effect may be lost when the drug is used in high doses, due to the simultaneous inhibition of platelet Thromboxane A_2 and vessel wall PgI_2 [20, 21]. High dose aspirin may therefore have been an unfortunate selection on our part, and further clarification of this problem may require low dose aspirin regimens, or the selection of different antiplatelet function drugs.

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Dr B J Boughton,
Senior Lecturer in Haematology
Haematology Department,
Queen Elizabeth Hospital,
Edgbaston,
Birmingham B15 2TH (England)

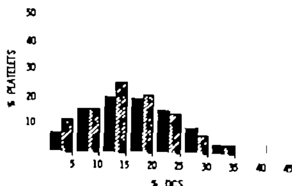


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means of the standard May-Grünwald-Giemsa method. Platelet size was evaluated by the use of micrometer applied to a standard microscope. Drumsticks and pseudodrumsticks were counted in thinly spread blood smears, stained with Giemsa. Drumsticks were considered only those nuclear appendages that appeared as darkly stained heads attached by thin stalk or filament to the nucleus [2]. All other appendages not meeting the above requirement were considered as pseudodrumsticks [2, 18]. Plasma and platelet B-thromboglobulin assays were carried out using the kit supplied by Amersham Laboratories, London. Serotonin ³H uptake and release was carried out by means of previously reported modification [8] of the method proposed by *David and Herson* [1]. The propositus is an 8-year-old male and was sent to us for *evaluation of mild thrombocytopenia. The* patient had been known to have mild thrombocytopenia for the past 5 years. The leukocytopenia was first noted after mumps. At that time bone marrow biopsy was carried out and found to be normal. Platelet counts varied between 100,000 and 150,000. Bleeding was mild and characterized by occasional epistaxis and easy bruising.

The second patient was 40-year-old male, father of the propositus. He has been asymptomatic all his life. The mother and the 2 sisters of the propositus wer also investigated. The family pedigree is reported in figure 1. No consanguinity as present in the family.

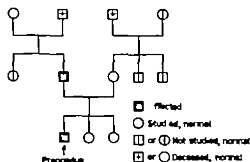


Fig. 1. Family pedigree. Both father and son were affected. All other members of the family investigated are normal. The parents of the propositus were not consanguineous.

Results

Results are summarized in tables I and II. WBC count was normal in both proposi-

Table I. Main features of leukocytes in the proposits

Test	Propositus	Father	Normal values
WBC count (per µl)	6,600	8,100	6,000–9,000
Differential count	normal	normal	–
Monocytes and lymphocytes	normal	normal	–
PMN size, µm	normal	normal	10–15
PMN segmentation			
Arneth formula (%)			
1 lobe	3	2	2
2 lobes	17	22	31
3 lobes	41	48	42
4 lobes	33	24	18
5 lobes	6	4	7
PMN granulations	normal	normal	–
PMN inclusions	no	no	no
Alkaline phosphatase stain	normal	normal	–
Peroxidase stain	normal	normal	–
Chloracetate esterase stain	normal	normal	–

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Increased Number of Pseudodrumsticks in Neutrophils and Large Platelets. A 'New' Congenital Leukocyte and Platelet Morphological Abnormality[1]

A. Girolami, F. Fabris, A. Casonato and M. L. Randi

University of Padua Medical School, Institute of 'Semeiotica Medica', Padua, Italy

Key Words. Large platelet · Leukocyte platelet abnormality · Nuclear appendages · Polymorphonuclear · Pseudodrumsticks

Abstract. 2 members of a family, a child and his father, showed a combined morphological abnormality of leukocytes and platelets. The abnormality consisted of the presence of pseudodrumsticks in the neutrophils and of large platelets. One or more than one pseudodrumstick was present in about 40% of neutrophils. Leukocyte count, differential count and enzymatic stains were normal. Large platelets constituted about 25% of the platelet population. 1 patient also had mild thrombocytopenia which appeared to be unrelated to the basic defect since it appeared after a parotitis infection. Platelet function was normal but for a moderate prolongation of the bleeding time in the patient who had mild thrombocytopenia. No chromosomal abnormality was present in the propositi. The condition seems different from other leukocyte and platelet abnormalities so far described.

Several congenital or acquired morphological changes of leukocytes have been described [3]. Associated anomalies of granulocytes and platelets are extremely rare. The only anomaly so far known is the May Hegglin anomaly [10, 13, 15] which consists of the presence of giant platelets and Döhle inclusion bodies in the leukocytes. The purpose of the present study is to describe what appears to be a 'new' combined abnormality of polymorphonuclear neutrophils and platelets.

This study was supported by grants from the MPI, Rome (grant 1952, 1979), from the CNR, Rome (grant 78.02123.04) and from the Venetian Region, Venice.

Material and Methods

Material and methods have been reported in detail elsewhere [4, 6, 7]. Only pertinent data will be supplied here. Histochemical staining for leukocyte alkaline phosphatase, esterase and peroxidase was carried out according to standard procedures. Chromosomal analysis was carried out for us by the Cytogenetics Service of the Pediatric Department of Padua University. Arneth's formula evaluation and drumsticks or pseudodrumsticks counts were carried out on smears stained by

means of the standard May-Grünwald-Giemsa method. Platelet size was evaluated by the use of micrometer applied to a standard microscope. Drumsticks and pseudodrumsticks were counted in thinly spread blood smears, stained with Giemsa. Drumsticks were considered only those nuclear appendages that appeared as darkly stained heads attached by thin stalk or filament to the nucleus [2]. All other appendages not meeting the above requirement were considered as pseudodrumsticks [2, 18]. Plasma and platelet B-thromboglobulin assays were carried out using the kit supplied by Amersham Laboratories, London. Serotonin ³H uptake and release was carried out by means of previously reported modification [8] of the method proposed by *David and Herson* [1]. The propositus is an 8-year-old male and was sent to us for evaluation of mild thrombocytopenia. The patient had been known to have mild thrombocytopenia for the past 5 years. The leukocytopenia was first noted after mumps. At that time bone marrow biopsy was carried out and found to be normal. Platelet counts varied between 100,000 and 150,000. Bleeding was mild and characterized by occasional epistaxis and easy bruising.

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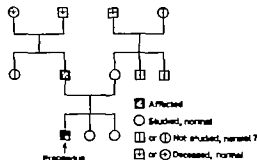


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University of Padua Medical School, Institute of 'Semeiotica Medica Padua, Italy

Key Words. Large platelet Leukocyte platelet abnormality Nuclear appendages Polymorphonuclear Pseudodrumsticks

Abstract. 2 members of a family a child and his father showed a combined morphological abnormality of leukocytes and platelets. The abnormality consisted of the presence of pseudodrumsticks in the neutrophils and of large platelets. One or more than one pseudodrumstick was present in about 40% of neutrophils Leukocyte count, differential count and enzymatic stains were normal Large platelets constituted about 25% of the platelet population 1 patient also had mild thrombocytopenia which appeared to be unrelated to the basic defect since it appeared after a parotitis infection. Platelet function was normal but for a moderate prolongation of the bleeding time in the patient who had mild thrombocytopenia. No chromosomal abnormality was present in the propositi. The condition seems different from other leukocyte and platelet abnormalities so far described.

Several congenital or acquired morphological changes of leukocytes have been described [3] Associated anomalies of granulocytes and platelets are extremely rare The only anomaly so far known is the May Hegglin anomaly [10 13 15] which consists of the presence of giant platelets and Döhle inclusion bodies in the leukocytes The purpose of the present study is to describe what appears to be a 'new combined abnormality of polymorphonuclear neutrophils and platelets.

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Material and Methods

Material and methods have been reported in detail elsewhere [4, 6, 7]. Only pertinent data will be supplied here. Histochemical staining for leukocyte alkaline phosphatase, esterase and peroxidase was carried out according to standard procedures. Chromosomal analysis was carried out for us by the Cytogenetics Service of the Pediatric Department of Padua University Arneth's formula evaluation and drumsticks or pseudodrumsticks counts were carried out on smears stained by

means of the standard May-Grünwald-Giemsa method. Platelet size was evaluated by the use of micrometer applied to standard microscope. Drumsticks and pseudodrumsticks were counted in thinly spread blood smears, stained with Giemsa. Drumsticks were considered only those nuclear appendages that appeared as darkly stained beads attached by thin stalk or filament to the nucleus [2]. All other appendages not meeting the above requirement were considered as pseudodrumsticks [2, 18]. Plasma and platelet B-thromboglobulin assays were carried out using the kit supplied by Amersham Laboratories, London. Serotonin ³H uptake and release was carried out by means of previously reported modification [8] of the method proposed by *David and Herion* [1]. The propositus is an 8-year-old male and was sent to us for evaluation of mild thrombocytopenia. The patient had been known to have mild thrombocytopenia for the past 5 years. The leukocytopenia was first noted after mumps. At that time bone marrow biopsy was carried out and found to be normal. Platelet counts varied between 100,000 and 150,000. Bleeding was mild and characterized by occasional epistaxis and easy bruising.

The second patient was 40-year-old male, father of the propositus. He has been asymptomatic all his life. The mother and the 2 sisters of the propositus were also investigated. The family pedigree is reported in figure 1. No consanguinity was present in the family.

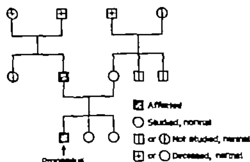


Fig. 1. Family pedigree. Both father and son were affected. All other members of the family investigated are normal. The parents of the propositus were not consanguineous.

Results

Results are summarized in tables I and II. WBC count was normal in both proposi-

Table I. Main features of leukocytes in the proposid

Test	Propositus	Father	Normal values
WBC count (per µl)	6,600	8,100	6,000–9,000
Differential count	normal	normal	–
Monocytes and lymphocytes	normal	normal	–
PMN size, µm	normal	normal	10–15
PMN segmentation			
Arneth formula (%)			
1 lobe	3	2	2
2 lobes	17	22	31
3 lobes	41	48	42
4 lobes	33	24	18
5 lobes	6	4	7
PMN granulations	normal	normal	
PMN inclusions	no	no	no
Alkaline phosphatase stain	normal	normal	–
Peroxidase stain	normal	normal	–
Chloracetate esterase stain	normal	normal	–

ti. Size and shape of polymorphonuclears appeared normal However on close exami nation an increased number of nuclear appendages or pseudodrumsticks were noted in the PMN (neutrophils and eosinophils) Lymphocytes and monocytes were normal in appearance The frequency of the presence of one or more than one drumstick in the PMN is reported in table II About 40% of the propositi s granulocytes showed at least one pseudodrumstick (fig. 2) The Arneth s formula was normal in both the propositi. The frequency of pseudodrumsticks was higher in PMN with 2–4 nuclear segments (fig. 3) The number of 'true drumsticks found in the mother and in the 2 sisters of the propositus were within normal limits (table III) Plasmatic clotting tests were all within normal limits. Platelet counts were slightly decreased in the propositus and perfectly normal in the father Platelet morphology studies showed the presence of both normal and large platelets (fig. 4) The large platelets with a diameter greater than 3 µm appeared to represent about 25% of the total (fig 5) Bleeding time was moderately prolonged in the propositus who had the mild thrombocytopenia but was normal in

Table II. Coagulation study in the propositi

Test	Case I	Case II	Normal values
Platelet number $\times 10^3/\mu l$	131	238	150–350
Bleeding time, min	7	3	<5
Platelet aggregation (ristocetin) %	60	74 6	72±12.7
Platelet adhesiveness, %	44.2	–	70±13.2
Serotonin ^3H uptake, %	75	77 4	80±6
Release, %	41 4	40 6	42±10
Plasma β-thromboglobulin, ng/ml	44	45	10–45
PRP β-thromboglobulin, ng/10^6 platelets	66	89.2	30–60
Clot retraction	complete after 10 h	complete after 6 h	complete within 10 h
Partial thromplastin time, s	38 1	35 2	30–42
Prothrombin consumption, s	32	38	>20
Prothrombin time, s	14	14	13–15
Factors II, V VII, VIII, IX, X, XI XII, XIII	normal	normal	60–160%

Table III. Distribution of drumsticks and pseudodrumsticks in the 2 propositi and in other family members

Patient	Number of PMN with pseudodrumsticks, %	True drumsticks, %	Number of PMN with more than one pseudodrumsticks
Propositus	42	0	20
Father	41	0	16
Mother	0 5	3	0
Sister	1	2.5	0
Sister	2	2	0

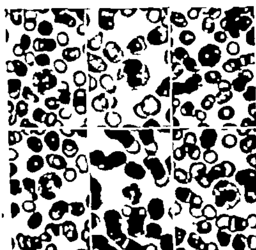


Fig. 2. Example of PMN with pseudodrumsticks. The upper row refers to the propositus, the lower row to the father. One or two pseudodrumsticks are seen in each PMN. The nuclear appendages are rather thick and short. No 'true' drumstick is evident.

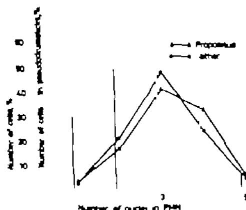


Fig. 3. Distribution of pseudodrumsticks according to PMN nuclear lobulation or segmentation. The highest percentage of pseudodrumsticks is noted in PMN with 2, 3 or 4 nuclear lobes. The number of cells with pseudodrumsticks entered (vertical bars) represents the average values observed in the 2 propositi.



Fig. 4. Example of large platelets in the propositus (upper row) and in the father (lower row). It may be noted that normal-sized platelets are also seen together with large platelets.

the father. Prothrombin consumption, platelet aggregation to ristocetin, platelet adhesiveness and serotonin ^{3}H uptake and release (collagen 2 μg/ml) were normal.

Discussion

The main features presented by the two propositi were: presence of nuclear appendages which appear to be pseudodrumsticks in the PMN and the presence of both normal and large platelets. About half of the PMN showed one or more than one pseudodrumstick. The largest number of pseudodrumsticks was present in the neutrophils with 2 or 3 nuclear segments. The PMN with only 1 or 5 nuclear segments had less pseudodrumsticks. The low percentage of pseudodrumsticks found in neutrophils with 5 nuclear segments is interesting in view of the fact that 'true' drumsticks have been reported to become more frequent with the increase of the nuclear segmentation in normal individuals and in the hypersegmenta-

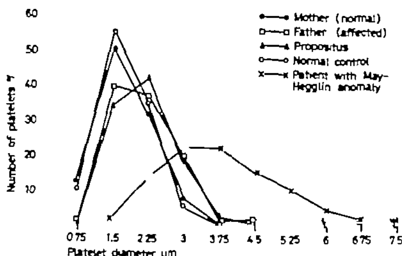


Fig. 5. Distribution of platelet sizes in the propositus, in the father (other affected patient), in the mother and in an unrelated normal subject. About 25% of the propositi's platelets are large, greater than 3 µm and up to 6–7 µm. For comparison the platelet size distribution curve of a patient with May Hegglin anomaly is also shown. The size of the large platelets seen in the propositi is similar to the size of the platelets seen in the May Hegglin anomaly

tion anomaly [3, 12]. No true drumstick was evident in the neutrophils of the propositi. All pseudodrumsticks were in fact rather thick and lacked the typical aspect of the pseudodrumstick [2].

Coarse nuclear appendages or pseudodrumsticks may occasionally be seen in normal subjects but never in such an elevated number [3]. That this is so is well demonstrated by the fact that unaffected members of the family showed only about 1% of coarse nuclear appendages. The presence of increased nuclear appendages has also been tentatively associated with carcinoma [9]. Our patients have no clinical or laboratory evidence for neoplastic disease. Finally an increased number of nuclear clubs and tags in neutrophils was described in patients given androgen therapy [14]. This does not apply to our patients, who never received such medications. As far as platelets are concerned, it is interesting to note that only about 25% of platelets are large, the rest were practically normal in size. However this is still significant since only an occasional large platelet is seen in normal subjects [5]. The size of the large platelet was similar to that observed in May Hegglin anomaly occasionally reaching 6 and 7 µm in diameter. In a normal subject platelets of that size are never seen. One of the propositi also had a mild thrombocytopenia. Whether this is significant or represents an associated anomaly remains to be clarified. Since identical morphological changes of leukocytes and platelets were present in the father who showed a normal platelet count, we tend to consider the mild thrombocytopenia found in the propositus as unrelated to the basic defect. In agreement with this interpretation is the apparently acquired nature of the thrombocytopenia in the propositus, which appeared after an acute parotitis. The presence of large platelets did not seem to significantly affect platelet function. Prothrombin consumption, serotonin release, ristocetin induced platelet aggregation and plasma β-thromboglobulin were normal. Platelet β-thromboglobulin was slightly elevated and this is in agreement with the presence of large platelets. Similar findings have in fact been seen in the May-Hegglin anomaly [18]. The presence of large platelets appears to be unrelated to platelet number since they were present in a similar pattern both in the propositus who had a mild -

Table IV Differential diagnosis between May-Hegglin anomaly and the present defect

Feature	May-Hegglin anomaly	Present defect
Hereditary pattern	autosomal dominant	autosomal dominant
PMN size	normal	normal
Arneth formula	normal	normal
PMN special feature	Döhle bodies	pseudodrumsticks
Platelet number	mild to moderate thrombocytopenia	normal (or mild thrombocytopenia?)
Platelet size	giant	normal or giant (about 25%)

thrombocytopenia and in his father who had a perfectly normal count. Whether these large platelets represent a separate population or a modification of the main normal platelet population remains to be clarified. The anomaly described here seems similar to the family described by *Seman* [17]. However in that case no large platelets were present and therefore a different defect is probably involved. It is interesting to note that in the patients described by *Seman*, the percentage of neutrophils with pseudodrumsticks or nuclear appendages was slightly higher than that observed in our patients, namely about 75% as compared to about 40%.

Increased nuclear appendages have also been described in association with D [13–15] trisomy [11 14 16] but this does not apply to our patients since they both had a normal chromosome pattern. The relation existing among these entities remains to be clarified. The anomaly also appears different from the other known associated defect involving leukocytes and platelets, namely May-Hegglin anomaly. The main differences between the two conditions are summarized in table IV.

In conclusion it seems very likely that the propositi present a hitherto unrecognized morphological abnormality of polymorphonuclear's and platelet's maturation.

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Prof. Antonio Girolami,
Istituto di Semeiotica Medica,
dell'Università di Padova,
Via Ospedale Civile, 105 Padova (Italy)

Short Communication

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Hyperglobulinemic Purpura in the Course of Multiple Myeloma

Meir Shalit, Shlomo Bar-Sela, Avigdor Leviatan and Yaakov Naparstek

Department of Medicine A and Department of Pathology, Hadassah University Hospital, Jerusalem, Israel

Key Words. Hyperglobulinemic purpura · Multiple myeloma

Abstract. Secondary hyperglobulinemic purpura of Waldenström is characterized by polyclonal gammopathy associated mainly with autoimmune diseases. Its occurrence with multiple myeloma is very rare. We describe a patient who developed characteristic lesions of hyperglobulinemic purpura in the course of IgA myeloma. Skin biopsy revealed deposition of IgA in the blood vessels.

The syndrome of benign hyperglobulinemic purpura (HGP) of Waldenström is characterized by purpura on the lower extremities, hyperglobulinemia, mild hypochromic anemia and elevated erythrocyte sedimentation rate [1]. Two types have been described: a primary form without an underlying disorder and a secondary form in association with autoimmune diseases and renal tubular acidosis [2–5].

The occurrence of HGP in the course of multiple myeloma (MM) is very rare and, to the best of our knowledge, only 2 cases have been reported [6, 7]. An additional case is described herein.

Case Report

A 65-year-old male was admitted to the department of Medicine A for recurrent purpura. 2 years prior to admission, while the patient was asymptomatic, monoclonal gammopathy IgA type lambda, was found. Bone lesions were not detected and bone marrow aspirate was not diagnostic for multiple myeloma. Total serum protein was 8.9 g/dl, albumin 4.4 g/dl, globulin 4.5 g/dl, IgG 830 mg/dl, IgM 108 mg/dl and IgA 1,089 mg/dl. 8 months prior to admission, purpuric rash appeared over the shins and calfs. Physical examination revealed pallor, the liver was palpable 2 cm below costal margin and marked purpura was seen over the shins and calfs (fig. 1). Significant laboratory tests were: erythrocyte sedimentation rate 120 mm/h, hemoglobin 12 g/dl, white blood cell count 7,100/mm³, differential count normal, platelet count 125,000/mm³. Bleeding time and clot retraction were normal but the capillary fragility was increased. Total serum protein was 9.9 g/dl, albumin 3.1 g/dl, globulin 6.8 g/dl, IgG 670 mg/dl, IgM 39 mg/dl and IgA 2,980 mg/dl. Urinalysis, liver and renal function studies, calcium, phosphorus and uric acid levels were all normal. Tests for cryoglobulin, LE cells, antinuclear antibodies, and latex globulin fixation were negative. A bone mar

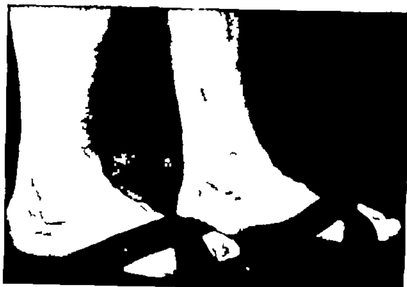


Fig. 1. Lower extremities of patient showing typical purpuric lesions.



Fig. 2. Biopsy of skin lesion showing mild perivascular infiltrate. HE. Original magnification × 125

row aspirate showed heavy infiltration with myeloma cells. No lytic lesions were detected by X-ray and radionuclide surveys of the bones. Circulating immune-complexes (C1q method) were detected. Biopsy of a purpuric lesion showed atrophy of the epidermis and a mild inflammatory infiltration of the upper dermis (fig. 2). Immunofluorescence disclosed positive staining for IgA in the wall of the blood vessels of the upper dermis (fig. 3)

Discussion

Secondary HGP has been described in association with polyclonal gammopathy mainly in patients with autoimmune disorders [2, 3] To the best of our knowledge only 2 cases have been reported in association with MM confirmed by the presence of paraprotein and plasma cell infiltration of the bone marrow [6 7] In one of these the paraprotein was not identified, while in the other it was IgG type kappa. 2 additional patients had the classical symptoms of HGP without *paraproteinemia, preceding the development of MM* [8 9]

The patient described by us developed HGP several months after the incidental detection of paraprotein in the blood. Purpura appeared only after a substantial increase in the concentration of IgA took place IgA was also the protein detected in the vascular -



Fig. 3. Immunofluorescent staining with anti-human IgA showing fluorescence in blood vessel walls of the upper dermis. Original magnification × 500.

lesions. Thus, a cause-effect relationship between the paraproteinemia and these lesions is very likely. The absence of cryoglobulin, the normal thrombocyte count and the absence of amyloid deposits in the blood vessels precludes the possibility that any of these was operative in the pathogenesis of the purpura.

The exact pathogenesis of HGP is still not clear. *Kunkel* et al. [10] described the presence of 'intermediate immune complexes' in their patients. It was further suggested that these immune complexes were composed of IgG and anti-IgG and that they were the hallmark of the syndrom [11, 12]. Whether these complexes cause blood vessel injury by mechanical obstruction or by an associated vasculitis is not clear.

Our patient manifested significant vascular deposition of IgA alone. There is no indication of the pathogenetic mechanism that caused the capillary damage in this case. The presence of purpura in MM is usually related to thrombocytopenia or cryoglobulinemia. Ou[r] case suggests that the possibility of HGP should be explored as well.

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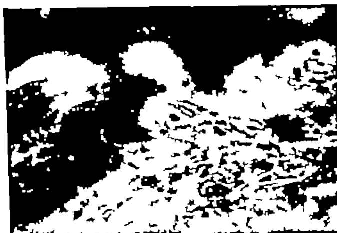


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Dr Meir Shalit, Department of Medicine A, Hadassah University Hospital, POB 12 000, Jerusalem (Israel)

Case Reports

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Lithium in Haematology A Case of Acute Intoxication

A Efira Y van Laethem M Ectors and J Unger

Saint Pierre Hospital, University of Brussels, Belgium

Key Words. Aplastic anemia Lithium, adverse effects

Abstract. A 74-year-old woman was treated by lithium carbonate 3 × 300 mg per day for drug-induced aplastic anemia. After 8 days, she suddenly developped severe impairment of consciousness with myoclonias and hypertonia which persisted during 10 days despite lithium withdrawal and sodium chloride infusion. Slight disorders of water and electrolyte metabolism, mild renal failure and the patient s age could have contributed to the development of intoxication. Short-term lithium administration may be life-threatening and should thus be prescribed cautiously in hematological disorders as in other conditions.

When lithium was used in the treatment of gout, as a sedative or as an anticonvulsant, severe side-effects were reported [1] Nevertheless, when lithium chloride was prescribed in the 50s as a substitute for sodium salt, the drug was described by the manufacturers as perfectly safe. Subsequently severe lithium poisoning, some times leading to death, occurred [2, 3] When lithium was recommended for sedation of psychotic excitement, its toxicity was recognized but minimized [4] and in 1950 the first death caused by lithium salts in psychiatry was reported by *Roberts* [5] From 1949 to 1975 more than 100 cases of lithium intoxication were reported [6], although psychiatrists follow strict therapeutic principles [3] Since 1975 lithium has been utilized in the treatment of granulocytopenic conditions, including Felty's syndrome, acute leukemia, granulocytopenia induced by cancer therapy and in the treatment of aplastic anemia [7] Since no case of severe toxicity had been reported *Stein et al.* [8] considered lithium to have limited toxicity only *Rothstein et al.* mentioned that, because of its adverse effects, lithium should not yet be accepted as a hematopoietic agent [9]

We report the first case of severe intoxication induced by lithium used in hematology

Report of a Case

A 74-year-old woman was admitted in March 1979 for cutaneous purpura and pallor. Hemoglobin was 7.1 g/dl, the white cell count, 1,600/ l,

with 10^9/ neutrophils the platelet count was 800/ μl. The bone marrow aspirate and needle biopsy of the iliac crest revealed hypoplasia affecting all cell lines, and no megakaryocytes. Oxphenylbutazone was suspected to be responsible for the pancytopenia. The patient received packed red cells and platelets. Mesterolone during 3 weeks was unsuccessful. Antibiotics were ordered for a urinary infection and were responsible for a slight diarrhea. Lithium carbonate 300 mg, 3 times a day was started on April 6. Plasma sodium level was 126 mEq/l, urea 11 mg/dl creatinine 0.62 mg/dl. On April 9 the lithium level in serum was 0.12 mEq/l. On April 14 the patient was confused and stuporous with fixed gaze, hypertonia, and myoclonias on tactile stimulation. Urea was 40 mg/dl creatinine, 1.4 mg/dl and sodium 126 mEq/l. Cerebrospinal fluid was normal. The lithium level in cerebrospinal fluid was 0.7 mEq/l on April 14. Plasma lithium level was 2.6 mEq/l. Lithium was withdrawn and sodium chloride infused. On April 16, the lithium level was 1.3 mEq/l and on April 17 0.8 mEq/l. Slow progressive improvement was observed and total recuperation obtained on April 24. Total blood count has been completely normal since April 12.

The role of lithium in blood recovery is unclear in this case, since slight monocytosis was already present 2 days before lithium administration.

Comment

Severe central nervous system toxicity occurred in an old woman with drug-induced pancytopenia after 8 days of lithium treatment and persisted during 10 days despite lithium withdrawal and sodium chloride infusion.

The encephalopathy presented by our patient could reasonably be attributed to lithium. The generalized neuromuscular irritability, protracted impairment of consciousness and absence of focal neurological signs have been described in various reports [2, 3, 10] as characteristic of lithium toxicity. The diagnosis was further suggested by the lithium levels in blood and cerebrospinal fluid. In blood, the lithium level was moderately above the upper limit of the therapeutic range (1.5 mEq/l) and the cerebrospinal fluid value of 0.7 mEq/l was comparable to the levels reported in other cases of lithium intoxication with drowsiness and coma [6]. There was no argument for an infectious etiology and the slight metabolic abnormalities could not account for the observed symptoms.

This case thus draws attention to the inherent dangers of lithium therapy even when a rather low dose is administered. Indeed, the typical oral dose used to reach a serum lithium level of 1.2–1.5 mEq/l is 1,200–3,600 mg daily [3]. Hyponatremia, renal failure, dehydration, may all have contributed to the rapid occurrence of lithium intoxication. In our case, the occurrence of severe symptoms despite moderately elevated lithium levels may have been, at least in part, related to the patient's age. An increased sensitivity to lithium with age was suggested by *Van der Velde* [10] who described acute toxic reactions to lithium in 3 elderly patients with serum lithium levels of less than 2 mEq/l. Whatever the results of further studies about lithium in the treatment of hematological disorders, physicians must be aware of the serious and sometimes life-threatening effects of this drug. Benefits must be balanced against potential risks.

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Dr A. Efira, Department Internal Medicine,
Saint Pierre Hospital, 322, rue Haute
B-1000 Brussels (Belgium)

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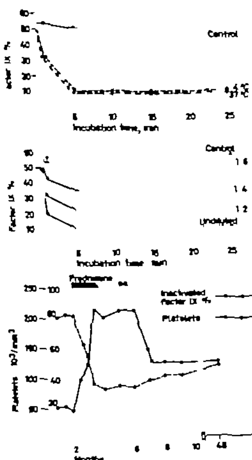


Fig. 1. Inactivation of factor IX by the inhibitor 0.2 ml of human serum diluted 1:10 in 0.9% saline was mixed with inhibitor containing sample (adsorbed serum) and after incubation several minutes at 37 C and 4 C factor IX activity was determined using one stage method. As control, the dilut serum was incubated with adsorbed normal human serum

b Potency of the inhibitor 0.2 ml Normal citrated human plasma which has been diluted to factor IX activity of human plasma, was mixed with various dilutions of patient' adsorbed serum (in saline) and the factor IX activity after several minutes of incubation was determined As control normal adsorbed serum was used instead of the inhibitor containing serum

not reveal any abnormalities in the small arteries.

The only altered laboratory tests were slight leukocytosis (12,000/mm³) with normal differential count, elevated alpha-2-globulin, positive Waaler-Rose test at 1/128 dilution and antinuclear antibodies in addition to slight decrease of the C_3 and C complement levels.

After the neurological episode and the arteriographic findings, diagnosis was changed to Takayasu's arteritis involving the left carotid and the left vertebral arteries and probably also involving, in diffuse manner the pulmonary arterial tree.

On the 20th hospital day routine coagulation study unexpectedly showed thrombocytopenia (50,000/mm³), prolonged whole blood clotting time (20 min, normal 6–8 min) and an abnormal kaolin-activated partial thromboplastin time of 92 (normal 30–40 s). Further studies revealed low levels for factor IX (9%) in low plasma dilution but normal levels when high dilutions were used.

The presence of an inhibitor was demonstrated by cross-mixing experiments: addition of normal plasma to patient' plasma in various proportions did not shorten the prolonged recalcification and partial thromboplastin times.

Factor IX activity of normal pooled plasma was lowered after short incubation with adsorbed patient's serum and plasma at 37 and 4 °C. Maximal inhibition occurred at 5 min after mixture and incubation (fig. 1) The factor IX inactivation capacity disappeared at 1:6 dilution (fig. 1b). Neutralization tests revealed that the inhibitor was of IgG nature with predominance of kappa chains.

A immunological pattern of thrombocytopenia was suggested by an abnormal indirect antiglobulin consumption test (75% of consumption) after the incubation of normal platelets with patient serum.

Effects of Corticosteroid Therapy on the Inhibitor Activity Thrombocytopenia and Clinical Course

After 45 days of treatment with prednisone (1 mg/kg/day) and 60 days of 20 mg every other day w observed gradual decrease in the anticoagulant activity (fig 1) and rise of the plate-

Evolution of the biological abnormalities under corticoid therapy

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Anti-Factor IX Circulating Anticoagulant and Immune Thrombocytopenia in a Case of Takayasu's Arteritis

A Torres J F Lucia A Oliveros C Vazquez and M Torres

Services of Hematology Neurology and Cardiology Social Security Hospital, Zaragoza, Spain

Key Words. Anti-factor IX Immune thrombocytopenia Takayasu s arteritis

Abstract. A 38-year-old male with proven Takayasu s arteritis presented, in a routine investigation, with thrombocytopenia, prolonged whole blood clotting time and activated partial thromboplastin time. Further studies demonstrated low levels of factor IX caused by a circulating anticoagulant. Immunological studies revealed an IgG (with kappa chains predominance) nature of this. Corticoid therapy decreased but did not suppress the anticoagulant activity Since similar coagulation abnormalities have been described in collagen diseases this observation is in support of this etiology to be considered in Takayasu s arteritis.

Takayasu s syndrome (TS) is an obliterative arteries of unknown origin mainly involving the aorta (arch and abdominal portion) and its branches [1–3] Acquired inhibitors against factor VIII or IX are rather infrequent findings in non-hemophiliacs and have mainly been described in patients with collagen diseases [5]

We report here the rare association of Takayasu s syndrome with circulating anti coagulant anti IX and immune thrombocytopenia.

Case Report

A 38-year-old caucasian male was admitted to our hospital because of shortness of breath Clinical findings, ECG pulmonary scan and cardiac catheterization allowed a diagnosis of primary pulmonary hypertension with cor pulmonale to be established.

15 days after admission the patient started to complain of weakness, fatigue, anorexia, frontal headache and depression Over the next 4–5 days he slowly developed a right homonymous hemianopsia paresthesias in the right arm that progressed to hemiplegia, mixed aphasia (mainly motor), and pain in the left side of the neck radiating to the head and face. EEG showed a focus of abnormal activity in the left temporal and parietal lobes. The left carotid artery was felt as being rigid and nonpulsatile. Arteriography of the right carotid showed marked shunting of blood through the anterior communicating artery Aortography: the left carotid did not fill at all. The origin of the left vertebral artery was slightly narrowed and the bronchial arteries were very dilated. A muscle biopsy taken from the right gastrocnemious did

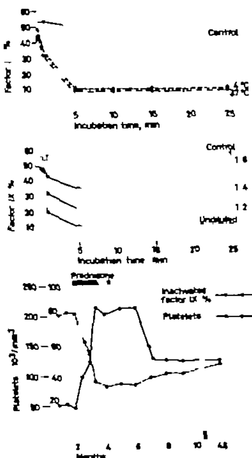


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Microcytosis of Unknown Origin

Klaus Neftel and Peter Zahler

Central Hematology Laboratory Inselspital, and Biochemical Institute of the University
Bern, Switzerland

Key Words. Biochemical membrane analysis Microcytosis

Abstract. We report a case of constant microcytosis which could not be related to any of the usual, known causes. The biochemical and functional investigations showed – apart from the small volume – no difference compared with normal erythrocytes. There was no evidence for a hereditary defect. It is concluded that the condition may represent a variant of the normal.

A decrease in the mean corpuscular volume (MCV) indicates congenital or acquired erythropoietic disorders, normally associated with various degrees of anemia. This paper reports a non-anemic case of constant microcytosis which could not be related to any of the known causes. It is concluded that this condition may represent a variant of the normal

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The 22 year-old patient as seen for the first time in December 1977 ith the clinical and biochemical features of hepatitis. The etiology remained unclear neither type A nor type B as demonstrable The tests for possible infectious agents er negative and the history gave no indication for toxic agent Liver biopsy was not performed. Serum transaminases and lkaline phosphatase decreased slowly and returned to normal values after 9 months.

On routine controls w observed constantly educed MCV together with an increased RBC count. As shown in table I, the other six routine tests carried out between March 1978 and May 1979 yielded normal erythrocyte values. I December 1977 determination of the same parameters by manual methods yielded the following values. RBC count (Neubauer chamber) 5.9 × 10⁶/µl, hematocrit (micromethod) 42 9 %, hemoglobin 14.5 g/dl, MCV 72 0 fl, MCH 24 6 pg, MCHC 34 1 The reticulocyte count was never increased (0 9–1 8 %). White cell and platelet counts were

Table I. Hemoglobin, hematocrit, RBC count and erythrocyte indices ranges between March 1978 and May 1979

Hgb g/dl	14 0	16 3
Hct, %	43.4	50 4
RBC 10⁶/µl	5.91	7.07
MCV fl	69	71
MCH, pg	23 0	23 7
MCHC,	32 7	34 0

let count. Similarly notable clinical improvement occurred. The neurological and the cardiopulmonary picture nearly disappeared leaving only slight motor aphasia, minimal brachial palsy and residual left nasal quadrantanopsia after 10 months.

Nevertheless, both the anticoagulation and the thrombocytopenia did not totally disappear persisting at abnormal levels 45 months after diagnosis.

Discussion

In extensive reviews [2, 3] no coagulation disorders have been reported in Takayasu's arteritis

The presence of an anti IX inhibitor in nonhemophilic patients is very rare *Shapiro and Hultin* [5] could collect in 1975 only 7 cases described in the world literature, neither of which had previously received factor IX. In our case as in the one described by *Sanchez Medal* et al. [4] and contrary to the other reported cases there was absolutely no bleeding tendency

The laboratory data and clinical course of our case confirm earlier findings. No clear answer to the question of etiology of the inhibitor or the influence of corticosteroids can be given

Acknowledgement

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A. Torres, Chief Service of Hematology
Assistant Professor of Medicine,
University of Cordoba Medical School,
Reina Sofia Hospital, Cordoba (Spain)

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Klaus Neftel and Peter Zahler

Central Hematology Laboratory, Inselspital, and Biochemical Institute of the University Bern, Switzerland

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MCHC, %	32.7	34.0

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A. Torres, Chief Service of Hematology,
Assistant Professor of Medicine,
University of Cordoba Medical School,
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The osmotic fragility of the patients' erythrocytes as decreased according to the microcytosis (50% hemolysis in 0.35% NaCl). The osmotic fragility of the parents' erythrocytes was normal.

A study of red cell survival was refused by the patient, but there were neither direct nor indirect signs of enhanced red cell turnover.

Porphobilinogen and δ-aminolevulinic acid in 24-hour urine sample were not increased. Erythrocyte 2,3-diphosphoglycerate (11.93 μmol/g hemoglobin) and ATP (5.00 μmol/g hemoglobin) ere within the normal range.

The oxygen dissociation curve was normal ith p50 = 24.8 mm Hg.

The filterability of the erythrocytes with filter-paper method [1] was also within normal limits. A therapeutic trial with 3 × tablets of 37 mg iron succinat during 2 months induced little increase in serum iron concentration but did not influence the microcytosis of red cells.

Biochemical membrane analysis

We compared the patient's erythrocytes with those from normal donor. Membranes were isolated using *Hamaguchi and Cleve* [2] method. Table II and figure 1 show the esults of lipid analysis and polyacrylamide-gel electrophoresis. The relatively low cholesterol and total lipid content may indicate relatively high content of free fatty acids and/or triglycerides. The phosphatide patterns of both samples are identical and resemble those published in the literature. Proteins and glycoproteins in the gel electrophoresis also show no divergence from normal red cell membranes.

Comment

The reported microcytosis persisted unaltered during an observation period of 1.5 years. The investigation proved that the phenomenon was not related to any known congenital or acquired disorder associated with microcytosis. Particularly the following conditions could be ruled out: iron deficiency sideroblastic anemia, thalassemia and other hemoglobinopathies, heavy-metal poisoning, enzyme deficiency and hereditary spherocytosis. *Bessman* [8] has recently reviewed 35 cases with an MCV of less than 70 fl but with elevated RBC counts. 26 of these had thalassemia minor and 4 had polycythemia vera. The remaining 5 had secondary polyglobulia with coincident iron deficiency after long-term blood loss, suggesting a higher frequency of this combination than expected. Such a combination is also excluded in the reported case. It might be of interest that the formula of England and Fraser discriminating between iron deficiency and thalassemia yielded values between –9.3 and –23.0 i.e. values clearly below those found by *Cunningham* [9] in 59 iron-deficient patients. Any correlation with the course of the hepatitis is rather unlikely. No evidence for a hereditary defect was apparent on examining the parents and siblings. The results of biochemical membrane analysis, erythrocyte filtration and O_2 binding studies are compatible with normal erythrocytes. This opinion is confirmed by the decreased osmotic fragility which reflects a higher surface/volume ratio of otherwise unaltered cells.

We conclude that the reported 'microcytosis of unknown origin' must be considered as an unusual variant of the normal.

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Table II. Phospholipid analysis of erythrocyte membranes

	Patient	Blood donor	Literature
Lipoid extraction (4) mg lipoid/mg protein (5)	1.05	0.90	
Cholesterol (6), % of total lipoid	29.5	32	
Total phosphorus (7), % of total lipoid	2.94	3.20	
Phosphatide distribution pattern, % of total phosphorus			
Lysophosphatidylcholin	1.38	1.38	1.62 ± 0.57
Sphingomyelin	25.39	25 92	25 75 ± 2.20
Phosphatidylcholin	31 73	30.79	30.56 ± 2.89
Phosphatidylserin	14 71	14 60	12.96 ± 3 09
Phosphatidylethanolamin	26.78	27.30	27 18 ± 3 01

normal as well. Besides constant microcytosis of the red cells, the morphological aspect of the blood smear was entirely normal. No spherocytes were present. The mean red cell diameter measured with an eye piece micrometer was 6.3 ± 0.4 μm (normal range: 7.2 ± 0.5 m).

During the acute phase of the hepatitis, a single value of serum iron was slightly decreased (47 μg/dl) whereas the four ensuing results were within normal limits (61–132 μg/dl) The iron-binding capacity was always normal The bone marrow aspiration contained relatively little but homogeneously stainable iron pigment. No abnormal sideroblasts were found. Hemopoiesis was unremarkable, the myeloid to erythroid ratio being 3 1

Hemoglobin electrophoresis showed a normal pattern (HbF = 0 34%, HbA = 2.5 %, no abnormal hemoglobin detectabl) Hemoglobin stability in the isopropanol test was normal The in vitro globin chain synthesis study gave normal results with an α/β ratio of 0.96 1.00 All of the following erythrocyte enzymes were found to be normal glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase, glutathione reductase, hexokinase, phosphohexose isomerase, phosphoglycerat kinase, phosphoglyceromutase, pyruvate kinase.

Blood from the parents of the patient also gave a normal hemoglobi electrophoresis. The fatherhood was proven by HLA-determination. Blood smears, peripheral cell counts and erythrocyte indices of the parents and siblings (1 sister and 1 brother) were all normal There were no spherocytes visible.

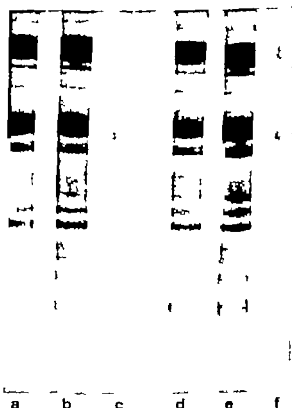


Fig. 1 Polyacrylamide-gel electrophoresis of the patient's red cell membranes (a–c) in comparison with normal red cells (d–f). Patient. a = 30 μg proteins, b = 45 μg proteins; c = 123 μg glycoproteins. Normal subject d = 30 μg proteins; = 45 μg proteins; f = 120 μg glycoproteins.

The osmotic fragility of the patients erythrocytes was decreased according to the microcytosis (50% hemolysis in 0.35% NaCl). The osmotic fragility of the parents' erythrocytes was normal.

A study of red cell survival was refused by the patient, but there were neither direct nor indirect signs of enhanced red cell turnover.

Porphobilinogen and δ-aminolevulinic acid in 24-hour urine sample were not increased. Erythrocyte 2,3-diphosphoglycerate (11.93 μmol/g hemoglobin) and ATP (5.00 μmol/g hemoglobin) were within the normal range.

The oxygen dissociation curve was normal with p50 = 24.8 mm Hg.

The filterability of the erythrocytes with filter-paper method [1] was also within normal limits. A therapeutic trial with 3 × tablets of 37 mg iron succinate during 2 months induced little increase in serum iron concentration but did not influence the microcytosis of red cells.

Biochemical membrane analysis

We compared the patient's erythrocytes with those from normal donor. Membranes were isolated using *Hamaguchi and Cleve's* [2] method. Table II and figure 1 show the results of lipid analysis and polyacrylamide-gel electrophoresis. The relatively low cholesterol and total lipid content may indicate relatively high content of free fatty acids and/or triglycerides. The phosphatide patterns of both samples are identical and resemble those published in the literature. Proteins and glycoproteins in the gel electrophoresis also show no divergence from normal red cell membranes.

Comment

The reported microcytosis persisted unaltered during an observation period of 1.5 years. The investigation proved that the phenomenon was not related to any known congenital or acquired disorder associated with microcytosis. Particularly the following conditions could be ruled out: iron deficiency, sideroblastic anemia, thalassemia and other hemoglobinopathies, heavy metal poisoning, enzyme deficiency and hereditary spherocytosis. *Bessman* [8] has recently reviewed 35 cases with an MCV of less than 70 fl but with elevated RBC counts. 26 of these had thalassemia minor and 4 had polycythemia vera. The remaining 5 had secondary polyglobulia with coincident iron deficiency after long-term blood loss, suggesting a higher frequency of this combination than expected. Such a combination is also excluded in the reported case. It might be of interest that the formula of England and Fraser discriminating between iron deficiency and thalassemia yielded values between −9.3 and −23.0, i.e. values clearly below those found by *Cunningham* [9] in 59 iron-deficient patients. Any correlation with the course of the hepatitis is rather unlikely. No evidence for a hereditary defect was apparent on examining the parents and siblings. The results of biochemical membrane analysis, erythrocyte filtration and O binding studies are compatible with normal erythrocytes. This opinion is confirmed by the decreased osmotic fragility which reflects a higher surface/volume ratio of otherwise unaltered cells.

We conclude that the reported microcytosis of unknown origin must be considered as an unusual variant of the normal.

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Klaus Neftel, Central Hematology Laboratory Inselspital CH–3010 Bern (Switzerland)

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